EFFECT OF OXYGEN ON THE EXPRESSION OF THE RESPIRATORY CHAIN IN MOUSE EMBRYO FIBROBLASTS

by

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ABSTRACT

The primary role of oxygen (O_2) in aerobic metabolism is its participation in the reaction catalyzed by cytochrome oxidase, the terminal electron transport complex in mitochondrial oxidative phosphorylation, but circumstantial evidence suggests that it may also have a role in regulating protein expression. This aspect has been most actively studied in bacteria and yeast. Some bacterial studies suggest that O_2 directly affects gene transcription, but studies of yeast indicate that O_2-associated effects on protein expression in this organism are mediated by the requirement for O_2 in the biosynthesis of heme.

O_2 also affects the expression of enzymes in the respiratory chain of mammalian cells. There may be a coordinate effect on the electron transport complexes or, alternatively, O_2 may have a specific effect on the expression of particular subunits (i.e., those translated in the mitochondrion or in the cytosol) or on heme-containing complexes. I focused my thesis studies on the following questions: (1) a decrease in O_2 availability to many mammalian cells leads to a decrease in the expression of cytochrome oxidase. Does O_2 affect the synthesis or assembly of all or particular subunits in this multi-subunit complex in cultured mammalian cells? (2) Does O_2 have similar effects on the expression of another respiratory heme-containing protein, cytochrome b-c_1?

Mouse embryo fibroblasts were grown in low (average medium O_2 tension, 7 torr) and control O_2 (average medium O_2 tension, 137 torr)

for 24h. Cells in both groups proliferated, carried on protein synthesis, and remained viable during the course of the experiments. In contrast, the effect of O_2 on cytochrome oxidase activity was more acute; cell-specific enzyme activity was only 55% of control in cells exposed to low O_2 for 24h. Relative to controls, there was a consistent reduction in the appearance of radiolabeled subunits in immunoprecipitates of cytochrome oxidase and cytochrome $\underline{b}$-c_1 prepared from low O_2 cells. Incorporation of radiolabeled amino acids into subunit I of cytochrome oxidase and the apocytochrome $\underline{b}$ protein of the $\underline{b}$-c_1 complex ranged from 51-100% of control, whereas the appearance of these pulse-labeled subunits in holoenzymes immunoprecipitated from low O_2 cells was in the range of 6-39% of control. The synthesis of subunit II of cytochrome oxidase by low O_2 cells ranged from 63-100% of control and assembly of this protein into the low O_2 immunoprecipitated enzyme ranged from 15-61% of control. Thus, the data suggest that O_2 had an effect on the assembly of these mitochondrially translated proteins that was independent of an effect on their synthesis.

CHAPTER ONE

INTRODUCTION

ATP is a universal and essential form of energy for cellular metabolism as we know it. A diversity of ATP generating pathways have been described encompassing three primary metabolic strategies: anaerobic, photosynthetic, and oxidative. The evolution and expression of enzymes specific to these pathways is a consequence of the availability of metabolites, reducing substrates and terminal oxidants.

Current theory postulates the prebiotic atmosphere of the earth was a mildly reducing mixture of carbon dioxide (CO_2), nitrogen (N_2) and water vapor, with only trace amounts of hydrogen (H_2) (Levine, 1988). The earliest forms of life to appear in the anoxic environment were likely procaryotes that derived energy from the conversion of chemical substrates to CO_2 and simple organic acids. From these progenitors, evolved anaerobic bacteria, rich in cytochromes and simple chlorophylls, that could utilize light energy to reduce CO_2 with a H_2 donor such as hydrogen sulfide, and carry on photophosphorylation. The photosynthetic bacteria gave rise to the blue-green algae with their more complex chlorophylls that use captured solar energy to split off hydrogen from water molecules (rather than hydrides) generating oxygen (O_2) as the necessary dehydrogenation product (Tappan, 1974).

It is now evident that photosynthetic production of O_2 was and is the predominant source of O_2 on earth (Levine, 1988). From geologic

and fossil evidence, it is believed that atmospheric O_2 levels were in the range of 0.2% about 670 million years ago, steadily rising to 21% about 400 million years ago, and remaining fairly close to that level to the present day (Cloud, 1983). The transition to an oxygenic environment was accompanied by the evolution of cells that could couple the phosphorylation of ADP with the sequential transfer of electrons through a series of enzyme-catalyzed redox reactions utilizing O_2 as the terminal oxidant.

Because of its highly favorable thermodynamic potential, its relatively low level of reactivity, its stability and solubility properties, O_2 is uniquely qualified as a terminal oxidant (George, 1965; Koppenol, 1988). In oxidative phosphorylation, the energy derived from the oxidation of NADH by O_2 is used to synthesize ATP. Whereas 36 moles of ATP are generated from the complete oxidation of one mole of glucose, only two moles of ATP are derived from the fermentation of one mole of glucose. Facultative microbes and invertebrates, such as parasitic helminths and bivalve molluscs, have evolved more efficient anaerobic pathways than fermentative glycolysis to produce ATP during periods of sustained anoxia, but the ATP yield is still considerably less than that of the aerobic pathway (Hochachka, 1980).

The evolutionary and functional significance of aerobic energy metabolism is apparent from the following observations:

(1) With few exceptions among described species, only certain procaryotes are strict anaerobes.

(2) Given a choice, facultative anaerobes preferentially use O_2 as the terminal electron acceptor and suppress the biosynthesis of enzymes required for anaerobic energy metabolism (Lascelles, 1964; Iuchi and Lin, 1988) or photosynthesis (Bauer, et al, 1988; Zhu and Hearst, 1988).

(3) The level of respiratory cytochromes present in the tissues of aerobic animals correlates with the energy demands or activity level of the tissue (Keilin, 1925).

Although O_2 is a preferred bioenergetic substrate for many organisms, its availability to the cell is often reduced because of (1) its limited solubility in aqueous environments; (2) the limited (whether because of disease, periodic changes in energy demand, or morphologic constraints) capacity of transporters and O_2 delivery systems to provide adequate O_2 to cells that are not in direct contact with the atmosphere; (3) changes in the availability of O_2 to the organism as a result of a change in habitat (for example, enteric bacteria or parasitic nematodes that cycle between aerobic and anaerobic environments during their lifetimes); and (4) periodic changes in O_2 availability to the environment (for example, estuarine mudflats that are alternately oxygenated and deoxygenated by changes in the tide).

All aerobic cells have retained the capacity to carry on some form of anaerobic metabolism to provide ATP during periods of reduced O_2 availability. Many cells can adapt to hypoxia or anoxia by turning on the synthesis of enzymes necessary for anaerobic energy production and repressing the synthesis of enzymes associated with oxidative

metabolism. If O_2 becomes available again, the synthesis of oxidative enzymes is induced and those associated with anaerobic metabolism are repressed. This effect of O_2 on the pattern of enzyme expression suggests that O_2 not only acts as the terminal electron acceptor in respiration and as a substrate in over 200 described enzymatic reactions (Keevil and Mason, 1978), but also regulates the biosynthesis of enzymes crucial to energy metabolism. Such an effect has been observed in bacteria, yeast, parasitic nematodes, higher plants and many mammalian cells.

Finding a mechanism for the effect of O_2 on enzyme expression has challenged investigators for over 40 years. Studies of bacteria have focused on the coordinate and reversible regulation of opposing enzyme systems (for example, the coordinate induction and repression of enzymes involved in anaerobic and aerobic respiration in response to O_2 availability). Much research activity has been directed at characterizing the effect of O_2 on the synthesis of respiratory cytochromes in yeast. A correlation between O_2 availability and the levels of oxidative and glycolytic enzymes has been found in many eucaryotic cells, including those of various mammalian tissues, but only a few studies have been reported describing the nature of the response at the molecular level. In the remainder of this review, I will briefly describe work that has been done, focusing on results that provide evidence for possible mechanisms for the O_2 dependence of enzymes involved in energy metabolism.

BACTERIA

Facultative bacteria produce energy by (1) oxidative phosphorylation, whereby ATP synthesis is coupled to electron transport reactions that utilize O_2 (aerobic respiration), nitrate, fumarate or trimethylamine N-oxide (anaerobic respiration) as the terminal electron acceptors; (2) photophosphorylation, that couples ATP formation to electron transport reactions that are driven by light and (3) fermentation, in which ATP is produced as a result of substrate level phosphorylations (Haddock and Jones, 1977). Enzymes specific to each of these pathways have been shown to be induced or repressed in bactera by O_2 availability. For example, transfer of cultures of Aerobacter aerogenes, Escherichia coli, Staphylococcus epidermidis, and Bacillus cereus from anaerobiasis to aerobiasis induces the synthesis of aerobic respiratory cytochromes (Moss, 1952; Moss 1956; Jacobs and Conti, 1965; Schaeffer, 1952). An increase in O_2 availability correlates with an increase in the activities of tricarboxylic acid (TCA) enzymes (Englesberg and Levy, 1955; Gray et al, 1966b) and with a decrease in the activities of key glycolytic enzymes (Engelsberg and Levy, 1955). The genes encoding nitrate, trimethylamine N-oxide, and fumarate reductases in E. coli are repressed in the presence of O_2, whereas the levels of enzymes in the TCA cycle, glyoxylate shunt, the pathway for fatty acid degradation , and the cytochrome o oxidase complex are repressed in the absence of O_2 (Iuchi and Lin, 1988). Synthesis of the enzyme nitrogenase in the nitrogen-fixing bacteria, Klebsiella pneumoniae, Rhizobium meliloti, and Rhodopseudomonas capsulata, is

induced under anaerobiasis (Dixon et al, 1988). Lowering the O_2 concentration in cultures of Rhodobacter capsulatus leads to an increase in the contents of the light harvesting and reaction center proteins and their respective mRNAs (Clark et al, 1984; Zhu and Hearst, 1986).

At least three general mechanisms have been proposed to account for the effects of O_2 availability on these enzymes: (1) the effect is mediated through an effect on heme availability; (2) changes in O_2 availability are sensed directly or indirectly by a regulatory protein that transduces the signal to a second protein that is capable of repressing or activating the transcription of genes encoding anaerobic or aerobic proteins; (3) O_2 availability affects the level of DNA supercoiling which in turn, coordinately and reversibly facilitates transcription of anaerobic and aerobic genes. A brief review of experimental work supporting these proposed mechanisms follows.

Effect of O_2 on Heme Availability in Bacteria

When S. epidermidis is grown anaerobically, it accumulates coproporphyrinogen (Heady et al, 1964). No cytochrome a and only traces of cytochromes b_1 and o (cytochrome o is a CO-binding b type cytochrome) are present in the bacterium. The addition of hemin (heme b) to anaerobic cultures restores the formation of cytochromes b_1 and o, but not a. Aeration of the culture induces the synthesis of all three cytochromes (Jacobs and Conti, 1965). This suggests the effect of O_2 on the expression of the respiratory cytochromes in this organism

is mediated by an effect on the availability of heme b and heme a. In this organism, as in eucaryotic cells, O_2 is likely a necessary substrate for the biosynthesis of heme prosthetic groups.

The effect of O_2 availability on heme biosynthesis is not a general phenomenon among the bacteria. Strict anaerobes and facultative anaerobes, such as E. coli, produce hemoproteins in the absence of O_2 (Lascelles, 1964; Gray et al, 1966a; Schaeffer, 1952). Alternate electron acceptors have been shown to substitute for O_2 in the coproporphyrinogen oxidase reaction (Tait, 1972), and a protoporphyrinogen oxidase (that does not react with molecular O_2 under physiological conditions and that behaves more like a dehydrogenase) has been isolated from an anaerobic bacterium, Desulfovibrio gigas (Klemm and Barton, 1987).

Effect of O_2 on Factors Involved in the Regulation of Gene Transcription in Bacteria

The presence of O_2 interferes with the induction of enzymes involved in anaerobic respiration in E. coli and Salmonella typhimurium. The repression of these enzymes in E. coli is thought to be mediated by a reduction in the effective concentration of the fnr gene product (Fnr), a soluble protein of M_r=31,000. Under aerobiasis, Fnr may exist in an inactive form, or alternatively, its synthesis may be repressed. In the absence of O_2, Fnr is expressed and positively activates the transcription of several genes, including those encoding nitrate, fumarate, and trimethylamine N-oxide reductases (Shaw and Guest, 1982; Iuchi et al, 1986).

The levels of seven Krebs cycle enzymes, the cytochrome o oxidase complex, and five other enzymes involved in aerobic metabolism are reduced in anaerobically grown E. coli. Recently, an arcA mutant has been observed to expess similar levels of all 13 oxidative enzymes in the presence or absence of O_2, although it retains the capacity to repress nitrate, fumarate, and trimethylamine N-oxide reductases when grown under aerobic growth conditions. This, along with evidence obtained from gene deletion mutants, suggests the arcA (arc is an acronym for aerobic respiration control) gene product coordinately regulates the synthesis of aerobic proteins by directly or indirectly repressing the transcription of each gene (Iuchi and Lin, 1988). It is proposed that Arc A, a soluble protein, may be free to interact with a membrane protein to sense O_2-induced physiological changes in the cell. For instance, Arc A might interact with a cytochrome and sense changes in the electrical potential or pH gradient across the cytoplasmic membrane, or, it might interact with ubiquinone to sense changes in the redox state of the electron transport chain. One result of these protein-protein interactions might be that Arc A becomes activated in the absence of O_2 to positively repress the transcription of aerobic genes. This mechanism implies that the functional state, rather than the expression, of Arc A is responsive to changes in O_2 availability. Evidence for the constitutive expression or stability of this protein is currently not available.

Nitrogenase is a nitrogen-fixing enzyme that is responsive to the availability of O_2. Synthesis of the enzyme is induced under anaerobic

conditions, when fixed nitrogen availability is limited, and is repressed in the presence of O_2, when fixed nitrogen is abundant. Regulation of nitrogenase synthesis in K. pneumoniae involves the interraction of several cis- and trans-acting factors, that are in turn regulated by the availability of O_2 and fixed nitrogen (Dixon, 1984).

Seventeen genes (referred to as nif for nitrogen fixation), organized in seven operons, are required for the synthesis and activity of nitrogenase in K. pneumoniae. The genes encode the three polypeptide subunits present in the assembled enzyme complex, several products involved in processing of these structural elements, two flavodoxins involved in the electron transport pathway to the nitrogenase, and two regulatory elements, nifL and nifA, that control transcription of each of the nif operons. The gene products of ntrA, ntrB and ntrC (NtrA, NtrB, and NtrC) are pleiotropic regulatory elements that control transcription of several genes involved in nitrogen metabolism, including the genes for nitrogenase and glutamine synthetase (an enzyme required for the assimilation of ammonia under limiting conditions of fixed nitrogen). The functional state of the NtrB protein is modulated by factors that sense the ratio of glutamine to 2-ketoglutarate present in the cell. This ratio reflects the cell's nitrogen metabolism status. Depending on its state, NtrB switches the NtrC protein between its repressor and activator forms by phosphorylating or dephosphorylating it. NtrA is a sigma factor that specifies transcription by core RNA polymerase on nif-specific promoters (Dixon, 1984 and refs therein; Ninfa and Magasanik, 1986; Gussin et al, 1986).

A current model of nif regulation in K. pneumoniae is as follows: Under conditions of limiting fixed nitrogen availability, NtrB activates NtrC; NtrC and NtrA activate transcription of the nifLA gene; the NifA protein, in the presence of NtrA, activates transcription of the remaining six nif operons, resulting in the synthesis and processing of nitrogenase (Gussin et al, 1986).

Mutant strains of K. pneumoniae that express nitrogenase in the presence of ammonia (an exogenous source of fixed nitrogen that normally suppresses endogenous nitrogen fixation) do not express nitrogenase in the presence of O_2 (Eady et al, 1978). This suggests the mechanism for repression by ammonia differs from that of O_2. The mode of repression by O_2, however, has not been elucidated. It is thought that O_2 repression may be mediated by a nif-specific gene. For instance, nifL may inactivate nifA in the absence of NtrC, and, as a consequence, prevent the synthesis of the nitrogenase (Dixon, 1984).

A similar system of control has been observed in the regulation of nitrogen-fixing genes in the symbiotic, obligate aerobe, R. meliloti. In this system, however, conrol of the nif regulon is independent of regulation by fixed nitrogen availability (via NtrC) and appears to be solely responsive to the availability of O_2. Thus, a decline in O_2 tension in the alfalfa root nodule, where this organism resides, may be the signal that triggers a cascade of regulatory genes to activate synthesis of nitrogenase (Ditta, et al, 1987; David et al, 1988).

Effect of O_2 on the Conformation of DNA in Bacteria

Yamamato and Droffner (1985) postulated that facultative anaerobes must carry environment-specific genes for growth in aerobiasis or anaerobiasis and that expression of these genes must be subject to global control by the stress imposed by each environment. Working with mutant strains of *S. typhimurium*, they demonstrated that DNA topoisomerase I activity was reduced or absent in strict anaerobic mutants and DNA gyrase was reduced or absent in strict aerobic mutants. The possibility that DNA exists in a supercoiled state under anaerobic growth conditions and in a more relaxed conformation under aerobiasis led to the proposal that DNA conformation may regulate the promoter activities of genes specific for aerobic or anaerobic respiration and growth. Support for this hypothesis was provided by the following observations: (1) the activities of superoxide dismutase and catalase (enzymes necessary to detoxify O_2 byproducts produced during aerobic metabolism) were comparable in the strict aerobic mutants and in wild type cultures grown in the presence of O_2 and (2) the activities of these enzymes were either insignificant or not detectable in the strict anaerobic mutants and in anaerobically grown wild type cultures.

Further evidence in support of Yamamoto and Droffner's hypothesis has been provided by studies of a facultative nitrogen-fixing bacterium, *R. capsulata*, and a purple nonsulfur bacterium, *R. capsulatus*, that is capable of chemotrophic growth in the presence of O_2 or phototrophic growth in the absence of O_2.

R. capsulata has an *nifR1* gene, that is homologous to the *ntrC* gene in *K. pneumoniae*, and three other genes (*nifR2*, *nifR3*, and *nifR4*) that are required for transcription of *nifHDK*, the operon that codes for the three polypeptides of nitrogenase. *NifR4* is analogous to *Klebsiella nifA*, but is itself repressed by ammonia. Kranz and Haselkorn (1986) reported (1) the isolation of 13 mutants of *R. capsulata* that are unable to repress *nif* gene transcription in the presence of ammonia, but that do repress transcription of an *R. capsulata nifH*:lac Z gene fusion when grown in the presence of O_2 or when grown anaerobically in the presence of an inhibitor of DNA gyrase, (2) that synthesis of the three nitrogenase polypeptides is induced in wild-type *R. capsulata* grown under anaerobiasis and limited available fixed nitrogen, but is repressed in similar cultures treated with an inhibitor of DNA gyrase, and (3) that anaerobic *K. pneumoniae* grown in the presence of a DNA gyrase inhibitor with limited available fixed nitrogen also fail to induce nitrogenase protein synthesis (the latter has been confirmed by Dixon et al, 1988). These results suggest that the presence of O_2 interferes with the conformation of DNA that directly or indirectly facilitates activation of *nif* gene transcription.

R. capsulatus obtains energy from oxidative phosphorylation when O_2 is available and from photosynthesis when O_2 levels are low. The proteins that catalyze aerobic or anaerobic energy metabolism in this organism are reversibly induced or repressed in response to changes in O_2 availability. The operons encoding the pigment containing protein complexes of the photosynthetic apparatus are all transcriptionally

repressed by molecular O_2. The *puf* operon (which codes for the light harvesting I polypeptides and two of the reaction center polypeptides) has an O_2-regulated promoter located 699 base pairs upstream from the *pufB* structural gene. This promoter region contains a sequence that closely resembles the consensus sequence recognized by the NtrA sigma factor on *nif*-specific promoters (Bauer et al, 1988 and refs. therein). The mechanism for the effect of O_2 on transcription of the photosynthetic proteins is unknown, but one notion is that the effect is mediated through O_2-induced changes in the conformational state of DNA. As in *S. typhimurium*, *K. pneumoniae* and *R. capsulata*, the evidence is circumstantial. Cultures grown under anaerobiasis show an increase in the levels of the photosynthetic proteins and their respective mRNAs, whereas neither the transcripts nor the translation products are induced in anaerobic cultures grown in the presence of DNA gyrase inhibitors (Zhu and Hearst, 1988).

EUCARYOTES

In eucaryotes, aerobic energy metabolism occurs in the mitochondria. Enzymes of the TCA cycle are localized in the mitochondrial matrix, and redox enzymes involved in electron transport are anchored in the mitochondrial inner membrane. Most of the polypeptide subunits comprising these enzymes are the products of nuclear genes and must be imported into the organelle during or after translation of their respective mRNAs in the cytoplasm. Depending on the species, 7-12 enzyme subunits are also encoded by mitochondrial

DNA. Vertebrates and yeast possess mitochondrial genes for the cytochrome *b* component of the cytochrome *b*-c_1 complex and subunits I, II and III of the cytochrome oxidase complex; in addition, vertebrate mitochondrial DNA encodes 7 of the 26-30 subunits present in the NADH dehydrogenase complex. The mitochondrial gene products are synthesized by a mitochondrial-specific protein translation apparatus. In animals and yeast, all of the RNA components necessary for the synthesis of mitochondrially-derived proteins are encoded by mitochondrial DNA, but the protein factors necessary for mitochondrial DNA transcription, mRNA translation, post-transcriptional processing and assembly of the various enzyme complexes are products of the nucleo-cytoplasmic synthesizing apparatus and must be imported at appropriate times. How the activities of the two genetic systems are coordinated is unclear (Attardi and Schatz, 1988).

Effect of O_2 on Yeast

In yeast, studies of O_2 effects on proteins associated with energy producing pathways have, for the most part, been limited to oxidative enzymes. This is perhaps because the effect of O_2 on these enzymes is so striking, and also because it has provided a means of studying the biogenesis of particular enzyme complexes.

Effect of O_2 on Yeast Mitochondria

Anaerobically-grown yeast do not respire, do not exhibit the characteristic absorption spectra of heme *a*, *b*, or *c*-type cytochromes,

and are deficient in many of the apoprotein subunits that comprise the cytochrome holoenzymes (Chin, 1950; Ross and Schatz, 1976; Woodrow and Schatz, 1979). This effect of altered O_2 tension on respiratory cytochrome content is reversible in that anaerobically grown yeast show de novo synthesis of the enzyme complexes upon oxygenation.

The loss of a functional respiratory chain in anaerobic yeast also results in the loss of well-defined mitochondrial inner membrane cristae. The latter phenomenon is likely the result of structural changes in the membrane resulting from the loss of protein. For example, the average liver mitochondrion has an outer membrane area of 1.9 μM^2 and an inner membrane area of 6.5 μM^2 (Schwerzmann et al, 1986). Integral proteins, including F_1-F_0 ATPase and five respiratory chain complexes represent 40% of the inner membrane mass and occupy up to 50% of the inner membrane area. There are approximately 2600 molecules of NADH dehydrogenase, 5400 molecules of the b-c_1 complex, and 15,600 molecules of cytochrome oxidase per rat liver mitochondrion (Schwerzmann et al, 1986). Given the density of proteins in and the relative size of the average inner mitochondrial membrane, it is easy to see that any disruption of protein content in this membrane would drastically alter the morphology of the organelle. The lack of structures with well-defined cristae in anaerobic yeast led some investigators to conclude the organelles were completely lost in the absence of O_2 (Wallace and Linnane, 1964). Later studies, however, demonstrated that mitochondrial particles (referred to as "promitochondria"), exhibiting F_1 ATPase activity and containing

mitochondrial DNA, were retained in anaerobic yeast (Criddle and Schatz, 1969). The effect of low O_2 tension, then, is not a general effect on the biogenesis of yeast mitochondria, but rather, is a specific effect on the expression of enzymes involved in oxidative metabolism. A change in the concentrations of these proteins in the mitochondrial membrane leads to obvious changes in the morphology of the organelle.

Effect of O_2 on the Heme Biosynthetic Pathway in Yeast

Many studies of yeast support the view that the effect of O_2 on the expression of the mitochondrial redox proteins is mediated by heme availability. In eucaryotes, heme is synthesized in mitochondria from succinyl coenzyme A and glycine in a series of eight enzyme catalyzed steps (Kurlandzka and Rytka, 1985 and refs. therein). O_2 is a substrate in the reactions catalyzed by the sixth enzyme, coproporphyrinogen oxidase, and the seventh enzyme, protoporphyrinogen oxidase (Mattoon et al, 1979). Heme is not detectable in mutants with lesions in the heme biosynthetic pathway, but is present at reduced levels in anaerobically grown wild type cells (Lukaszkiewicz and Bilinski, 1979; Zagorec and Labbe-Bois, 1986). The reason for the persistence of some heme under anaerobic growth conditions is not clear. The requirement for O_2 by coproporphyrinogen oxidase can be substituted for by NAD^+ or $NADP^+$ if ATP, methionine and a divalent metal are present (Poulson and Polglase, 1974), but nothing has been found to substitute for O_2 in the protoporphyrinogen oxidase reaction (Mattoon et al, 1979). The fact that it is technically difficult to

exclude all O_2 from yeast cultures may partially account for the presence of low levels of heme in anaerobic yeast (Mattoon et al, 1979; Zagorec and Labbe-Bois, 1986; Zagorec et al, 1988).

Under very low O_2 tensions, there is a good correlation between the level of coproporphyrinogen oxidase activity and heme content, suggesting that this enzyme is the rate limiting step when O_2 levels are sufficiently reduced. Enzyme and mRNA contents are increased in anaerobically grown yeast and in heme-deficient mutants. Supplementation of the heme mutants, but not the anaerobic cultures, reverses the effect of O_2 on the enzyme. This suggests the effects of O_2 and heme are independent. It is possible, however that anaerobic cells have an impaired ability to take up exogenous heme (Zagorec and Labbe-Bois, 1986). The significance of the elevated expression of coproporphyrinogen oxidase under anaerobiasis may be that it represents a means of upregulating heme production under conditions of limiting substrate availability (Zagorec et al, 1988).

Transcriptional regulation of the HEM13 gene coding for coproporphyrinogen oxidase is mediated by DNA sequence(s) located upstream of the initiation codon (Zagorec et al, 1988). There are two regions within this site that show homology to other O_2-responsive upstream regulatory sequences. One is a consensus sequence similar to a conserved sequence in the *nif* promoter (see above) and the other is a region that shows strong homology with the upstream activation site (UAS_1) of the CYC1 gene encoding iso-1-cytochrome *c* (see below). CYC1 transcription is activated when the HAP1 protein binds to the UAS_1 in response to heme and O_2 availability (Guarente and Mason, 1983;

Arcangioli and Lescure, 1985; Pfeifer et al, 1987). It remains to be determined whether the homologous sequences of the HEM13 gene are regulated by factors similar to NifA or HAP1.

Effect of O_2 on Cytochrome Oxidase in Yeast

Cytochrome oxidase (COX) in yeast is composed of 9 polypeptide subunits (Power et al, 1984). The largest subunits, I, II, and III, are translated in the mitochondria and the remaining subunits are translated in the cytosol. Heme a is an essential prosthetic group in the assembled complex.

Anaerobiasis has two separate effects on the expression of cytochrome oxidase in Saccharomyces cerevisiae: it severely depresses the content of individual mitochondrially-derived and nuclear-derived subunits and it interferes with the assembly of a functional enzyme complex (Woodrow and Schatz, 1979).

Western blots probed with antisera specific for COX subunits II, IV, V, VI and VII indicate the steady state levels of each of these subunits in promitochondria is less than 8% of their respective levels in aerobic mitochondria. The reduction in COX subunit content may be due to either decreased synthesis or decreased stability of the polypeptides in anaerobic yeast. The results of one experiment suggest that O_2 availability affects subunit synthesis. Anaerobically-grown yeast incorporate 19-50 times less radiolabeled methionine in subunits I, II and III than do aerated cells (Woodrow and Schatz, 1979). Since the cells in this experiment were harvested immediately after the

pulse, effects on subunit stability should have been minimal.

Recent reports have documented the existence of two isoforms of COX V that are equally functional in the assembled COX complex (Cumsky et al, 1985; Trueblood and Poyton, 1987). Oxygen (and heme) affect the transcription of COXV such that $COXV_a$ is transcribed more efficiently than $COXV_b$ in aerated (or heme-supplemented, anaerobic) cultures; $COXV_b$ is the predominant transcript in anaerobic cells (Trueblood, et al, 1988; Hodge et al, 1989). One proposal is that opposing regulatory actions of the HAP2 and REO1 (ROX1) gene products coordinate the transcription of these genes in heme-sufficient and heme-deficient cells (Trueblood et al, 1988).

Although the absolute levels of individual subunits of cytochrome oxidase are reduced in anaerobic yeast, total synthesis of the subunits is not abolished. Woodrow and Schatz (1979) took advantage of this to demonstrate the effect of O_2 on the assembly of individual subunits into a multi-subunit complex. Anaerobically grown yeast were divided into two aliquots. One aliquot was returned to growth under N_2, the other was transferred to growth in air. Each aliquot was labeled and chased in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, for a total of 45 minutes. Analysis of the mitochondrial translation products indicated no demonstrable differences in the amounts of subunits I, II, and III synthesized under these conditions. However, immunoprecipitation of isolated promitochondria with antisera directed against subunits VI and VII showed that O_2 was required to coprecipitate subunits I and II. The

requirement for O_2 in heme synthesis and the observation that heme deficient mutants continue to synthesize low levels of individual COX subunits, but fail to assemble them into a multi-subunit complex (Saltzgaber-Muller and Schatz, 1978) suggest that the effect of O_2 on COX assembly is secondary to its effect on heme synthesis. Another possibility is that the oxidation state of heme a or copper has an effect on enzyme assembly (Woodrow and Schatz, 1979).

Effect of O_2 On Cytochrome c in Yeast

Cytochrome c is a soluble protein that transfers electrons from the cytochrome b-c_1 complex to cytochrome oxidase during oxidative phosphorylation. Attachment of a heme moiety to the apoprotein is required for its import into the mitochondrion (Dumont et al, 1988) and for its subsequent activity in respiration. The nuclear genes, CYC1 and CYC7, code for two isoforms, iso-1 and iso-2-cytochrome c, respectively. The two proteins share 84% amino acid sequence homology and function interchangeably in electron transfer. Iso-1 cytochrome c comprises 95% of the available cytochrome c present in aerobic yeast. In anaerobically-grown yeast, iso-2-cytochrome c becomes the dominant species because a small amount of apo-iso-2-cytochrome c is still synthesized, whereas synthesis of apo-iso-1-cytochrome c under these conditions is not observed (reviewed in Sherman and Stewart, 1971).

O_2, heme and catabolite repression influence the levels of cytochrome c expressed in yeast. In general, one can sort out the effects of catabolite repression from those brought about by heme and

O_2 availability because the relative effects of these factors are similar whether analyzed in repressed or derepressed yeast. The effects of heme and O_2, however, are so interdependent that it has been difficult to dissociate the effect of one from the other.

Compelling evidence that the effect of O_2 on the synthesis of cytochrome c is mediated by heme was provided by the following experiment. Wild-type yeast and heme-deficient (heml) mutants were transformed with a plasmid containing a CYC1:lacZ gene fusion and were grown in the presence or absence of O_2. B-galactosidase production was substantially reduced in anaerobic wild type yeast and in the heml mutants. Supplementation of the cultures with the heme analogue, deuteroporphyrin, caused a 60-75 fold increase in B-galactosidase production in the heml mutants whether they were grown with or without O_2, and a 12 fold increase in B-galactosidase production in anaerobically grown wild type cells (Mason et al, 1983).

The CYC1 and CYC7 genes are transcriptionally activated in the presence of O_2 (Guarente and Mason, 1983; Pfeifer et al, 1987; Prezant et al, 1987; Zitomer et al, 1987). Available evidence indicates the effect of O_2 is mediated by heme. Heme-induced expression of CYC1 is coordinated by the binding of the HAP1 (CYC1) protein to an upstream activation site (UAS_1) (Pfeifer et al, 1987) and perhaps, by the binding of the HAP2/HAP3 complex at a second site (UAS_2) (Guarente et al, 1984; Hahn and Guarente, 1988). The role of heme in the transcriptional regulation of these genes is unknown. Heme may bind directly to HAP1 or act on another protein that subsequently alters HAP1 in some way (Pfeifer et al, 1987). The CYC7 gene has a heme-

induced regulatory site that is activated by HAP1 (Prezant et al, 1987; Zitomer et al, 1987) and a heme-repressed site that is regulated by ROX1 (REO1) (Lowry and Zitomer, 1988; Hastings and Zitomer, in preparation).

It is possible that CYC1, CYC7, $COXV_a$, $COXV_b$, and COXVI are members of a family of heme and/or O_2 responsive genes whose expression is coordinated by a common set of trans-acting factors. For example, ROX1 (which is induced in the presence of O_2 or heme-sufficient conditions) represses the transcription of genes such as CYC7, $COXV_b$, and possibly HEM13, in aerobically grown yeast. The preferential expression of these genes in anaerobic yeast may occur because the repressor protein, ROX1, is not made or is unstable in the absence of O_2 when heme is deficient (Lowry and Zitomer, 1988).

In contrast, the HAP family of gene-regulatory proteins appears to coordinate the transcription of genes that are preferentially expressed under aerobic conditions. As discussed above, heme, HAP1, and possibly, HAP2, and HAP3 are involved in transcriptional regulation of the CYC1 gene. Recently it has been shown that heme and HAP2 also regulate the transcription of $COXV_a$ and COXVI (Trueblood et al, 1988; Hodge, et al, 1989; Trawick et al, 1989).

Effect of O_2 on Other Eucaryotes, Including Mammalian Cells

Ascaris suum is a parasitic porcine nematode. The adult lives in the the O_2-deprived lumen of the small intestine and obtains energy from anaerobic respiration using electron acceptors, such as fumarate.

The eggs and early larval stages develop in the presence of O_2 and produce ATP from oxidative phosphorylation. Adults retain mitochondria, but lack a functional TCA cycle and have barely detectable levels of cytochrome oxidase activity. The specific activities of enzymes of anaerobic metabolism, including fumarate reductase, are increased in the adult. In contrast, L2 stage larvae have an active TCA cycle and cytochrome oxidase. The L2 larvae are infective, and when swallowed by an appropriate host, migrate, first to the lungs, and finally, to the intestine, undergoing a series of molts to the L4 and adult stage during the migratory passage. The transition from aerobic to anaerobic metabolism occurs between the L3 and L4 molts. Interestingly, L4 larvae isolated directly from porcine intestine do not exhibit cytochrome oxidase activity, whereas L4 larvae cultured in the presence of 5% O_2, 85% N_2, 10% CO_2 retain levels of cytochrome oxidase activity similar to those present in L2 larvae. This suggests that O_2 affects the expression of cytochrome oxidase in this organism (Komuniecki and Vanover, 1987).

Twenty proteins are induced when maize primary roots are deprived of O_2, for instance during flooding. Simultaneous with the induction of the anaerobic polypeptides (ANP), there is a repression of the synthesis of aerobic proteins. The identified ANPs are all enzymes involved in fermentative metabolism and include the two alcohol dehydrogenases (AdhI and AdhII), glucose phosphate isomerase, pyruvate decarboxylase, sucrose synthase, and aldolase. The promoters of the Adh genes and the aldolase gene are anaerobically activitated and share regions of sequence homology with each other and with a region of the

sucrose synthase promoter. It is suggested that the other ANPs may contain similar control sequences, and that a common trans-acting protein may regulate all of the anaerobically induced genes. The role of O_2 in the process has not been elucidated (Sachs et al, 1980; Rowland and Strommer, 1986; Dennis et al, 1988 and refs. therein).

Reciprocal changes in the expression of enzymes in the bioenergetic pathways of cultured mammalian cells have been shown to occur in response to changes in extracellular O_2 tensions. The activities of enzymes catalyzing glycolysis are increased under relatively low O_2 tensions (5-30 torr) and decreased under relatively high O_2 tensions (90-120 torr) (Robin et al, 1984; Wölfe and Jungermann, 1985). In contrast, the activities of enzymes in the mitochondrial TCA cycle and the electron transport chain decrease under relatively low O_2 tensions and increase under relatively high O_2 tensions (Pious, 1970; Simon et al, 1977; Simon et al, 1978; Hare and Hodges, 1982; Murphy et al, 1984). The O_2-induced changes in the activities of cytochrome oxidase, cytochrome $\underline{b}$ and cytochrome $\underline{c} + \underline{c}_1$ and of phosphofructokinase and pyruvate kinase (the latter two are glycolytic enzymes) have been shown to be due to changes in concentration (Pious, 1970; Hare and Hodges, 1982; Hance et al, 1980 Ptashne et al, 1983). The reciprocal pattern of O_2-induced changes in enzyme expression has been observed in proliferating and non-proliferating cells derived from distinct tissue types: alveolar and peritoneal macrophages, skeletal muscle myoblasts, and hepatocytes.

The changes in enzyme profiles are not immediate, but occur after hours of exposure to an altered O_2 tension.

In vivo studies support the observations made in cultured cells. The specific activity of cytochrome oxidase in chick embryo ventricles and the metabolic rate of the whole embryo is dependent on the availability of O_2 to the egg (Stock et al, 1985; Stock and Metcalfe, 1987; Asson-Batres et al, 1989). Histochemical analyses of mammalian liver show higher concentrations of succinate dehydrogenase (a TCA cycle enzyme) and cytochrome oxidase in the peripheral regions of liver lobules (where O_2 tensions are relatively high) than in central regions of the tissue (where O_2 tensions are relatively low) (Schumacher, 1957). The activities and contents of succinate dehydrogenase and cytochrome oxidase are higher, and the activities of pyruvate kinase and phosphofructokinase are lower in isolated alveolar macrophages (which experience *in vivo* O_2 tensions of about 100 torr in lungs at sea level) than they are in isolated peritoneal macrophages (which experience *in vivo* O_2 tensions of about 5 torr) (Simon et al, 1977; Dannenberg et al, 1963). Consistent with these observations, the rates of O_2 consumption are greater in periportal regions of the liver and in alveolar macrophages than they are in pericentral regions of the liver or in peritoneal macrophages (Matsumura and Thurman, 1983; Oren et al, 1963).

Mechanisms for the Effect of O_2 on Other Eucaryotes

Little has been done to characterize a mechanism for the effect of O_2 on the expression of bioenergetic enzymes in metazoans. Two

reported studies, with conflicting results, provide some evidence for the effect of O_2 on the expression of cytochrome oxidase in cultured mammalian cells. One study indicates that induced expression of cytochrome oxidase in proliferating HeLa cells requires translation of mitochondrial, but not cytoplasmic proteins (Pious, 1970). The other study demonstrated that the cell-specific level of cytochrome oxidase (heme a) in isolated hepatocytes increases during adaptation of the cells to culture in room air, and the increase is not blocked in the presence of chloramphenicol, an inhibitor of mitochondrial protein translation (Hare and Hodges, 1982).

Recently, it has been reported that transcription rates of the fermentative enzymes, lactate dehydrogenase, pyruvate kinase, triosephosphate isomerase and aldolase, are increased 2-5 fold during a 72 hour exposure of rat skeletal muscle (L8) myoblasts to 2% O_2 as compared with control cells maintained at 20% O_2 (Webster, 1987).

The role of heme in the biogenesis of mammalian cytochromes has been the subject of two published studies. As in yeast, it appears to have two functions: (1) an involvement in the synthesis of the apoproteins and (2) an involvement in the assembly of holoenzyme.

The maximal rate of incorporation of [^{3}H] ALA into heme isolated from the mitochondria of fetal rat liver is observed within 10 min following pulse-labeling of the mother. Subsequently, there is a rapid decrease in the rate of labeling, suggesting rapid turnover of mitochondrial heme in fetal liver. The rate of [^{14}C] leucine incorporation into fetal liver mitochondrial proteins is reduced to 75%

of control levels within 30 minutes of administration of inhibitors of ALA dehydratase or ferrochelatase (heme biosynthetic enzymes) to the mother. This effect on mitochondrial protein synthesis is partially reversed by supplementation of the mother with hemin (heme b). More specifically, the rate of incorporation of [^{14}C] leucine into cytochrome oxidase is reduced to less than 25% of control levels within 30 minutes of inhibition of heme biosynthesis. The effect on cytochrome oxidase synthesis can be reversed within 15 minutes by supplementation of the mother with heme. In these experiments, the functional activity of cytochrome oxidase parallels the heme-induced effects on enzyme synthesis: it decreases when heme is deficient and increases when heme levels are sufficient. Cycloheximide completely blocks *de novo* synthesis of cytochrome oxidase apoproteins within 45 minutes of its administration to the mother rat, but does not block assembly of precursor pools of apoproteins still present in the fetal liver cells. These results suggest that heme has two effects on the expression of cytochrome oxidase in fetal rat liver: (1) it affects either the transcription or translation of subunits coded for by nuclear DNA and (2) it affects the assembly of the holoenzyme (Woods, 1977). Another study has shown that heme *a*, but not heme *b*, induces the assembly of the three mitochondrially-translated subunits of cytochrome oxidase in isolated rat liver mitochondria (Wielburski and Nelson, 1984).

A study of whole chicken blood indicates the synthesis of coproporphyrin, protoporphyrin, and heme is differentially regulated by the availability of O_2. Heme synthesis is depressed when O_2 levels are

less than 7% and is not observed when available O_2 is less than 1% (Falk et al, 1959). These levels of O_2 are well within the physiological range and suggest that heme metabolism may be jeopardized in O_2 deficient tissues *in vivo*.

OVERVIEW OF THE EFFECT OF O_2 ON THE EXPRESSION OF BIOENERGETIC ENZYMES

Does O_2 regulate the expression of bioenergetic enzymes? Examination of the evidence from studies of species ranging from bacteria to mammals would suggest that it does, but that there is no reason, at present, to believe that it directly affects gene transcription, translation, or enzyme assembly. Models constructed thus far, predict the effect is mediated by some intervening factor, e.g., via a DNA binding protein, DNA conformation, heme, the redox state of the cell, or some other physiologically-responsive variable.

Is there a common pathway of regulation of O_2-responsive proteins? Before answering this question, it is perhaps worthwhile to consider why or whether one should expect the existence of a global regulatory mechanism. From one perspective, it seems foolish to expect that organisms as diverse and as specialized as nitrogen-fixing bacteria, yeast, parasitic nematodes, clams, maize seedlings and mammals should have retained similar patterns of regulation over gene expression or enzyme assembly. The diversity of regulatory mechanisms that have been described or postulated may be an affirmation of this. On the other hand, the amazing sequence conservation of bioenergetic proteins, such

as cytochrome *c* (reviewed in Lemberg and Barrett, 1973), argues there is every reason to expect conservation of regulatory mechanisms for expression of some proteins.

The transcriptional regulation of heme proteins and proteins associated with the heme biosynthetic pathway in yeast may turn out to be similar to the cascade regulatory mechanisms that control the expression of genes in bacteria. The control of gene expression by O_2-induced changes in DNA conformation is also a potential means of global regulation. No one has looked to see if this mechanism could explain the induction or repression of anaerobic and aerobic proteins in eucaryotes. Finally, heme appears to be a common O_2-responsive intermediary that affects the expression of bioenergetic enzymes in many aerobic organisms.

Control mechanisms similar to those found in yeast do exist in mammalian cells, but whether they are activated by changes in O_2 availability has not been determined. Deletion analysis of the rat somatic cytochrome *c* gene has revealed the presence of three sequence elements that are required for maximal expression of the gene. One of the regions contains two consensus CCAAT boxes (Evans and Scarpulla, 1988). The yeast HAP2/HAP3 complex recognizes CCAAT-containing transcription elements and is functionally interchangeable with a human DNA binding protein, CP1, that also recognizes CCAAT-containing sequences (Chodosh et al, 1988). A role for CP1 in regulating mammalian cytochrome *c* gene promoters has not been found, but the evidence suggests there is a likelihood for such regulation.

CHAPTER TWO

THESIS OBJECTIVES

DEFINITION OF THE THESIS PROBLEM

O_2 availability affects the expression of cytochromes that participate in oxidative phosphorylation in prokaryotes, unicellular eukaryotes and cultured mammalian cells (see Chapter One for a review and relevant references). All of these cells make substantially less of at least one cytochrome when grown under relatively low O_2 tensions (anaerobiasis or < 25torr) than they do when grown under relatively high O_2 tensions (120-150torr). Much work has been done to characterize the biochemical and genetic nature of this effect in bacteria and yeast, but only two studies, with conflicting results, have been reported regarding the mechanism for the effect in cultured mammalian cells (reviewed in Chapter One). The response of bacteria (*Aerobacter aerogenes*, *Escherichia coli*, *Staphylococcus epidermidis*, and *Bacillus cereus*) and yeast (*Saccharomyces cerevisiae*) is physiologically relevant because the levels of O_2 used in the experiments (anaerobiasis and aerobiasis) are levels that encompass the normal physiological range of O_2 for these organisms. The physiological significance of the response in cultured mammalian cells is difficult to assess because of the non-physiological nature of the laboratory culture conditions.

There is a broad range of O_2 tensions in mammalian tissues *in vivo*. At sea level, the O_2 tension range of human adult blood is 40-105torr (Vander et al, 1975) and that of human umbilical cord blood at birth is in the range of 4-48torr (Cunningham et al, 1989). Micropolarographic measurements of local tissue O_2 tensions (measured *in situ*) indicate the O_2 tension distribution in various mammalian tissues ranges from less than 1 to over 100 torr (from Kessler, 1974 as reported in Lubbers, D.W., 1983).

Given the wide range in O_2 tensions both within and among mammalian tissues and the relative hypoxemia of the fetus, one might expect to see a correlation between O_2 availability and the cellular content of the respiratory cytochromes *in vivo*. The results of several studies support this prediction: (1) histochemical analyses have demonstrated higher concentrations of cytochrome oxidase in the peripheral regions of mammalian liver lobules (where *in vivo* O_2 tensions are relatively high) than in central regions of the tissue (where O_2 tensions are relatively low) (Schumacher, 1957); (2) alveolar and peritoneal macrophages originate from a common precursor cell in the bone marrow, but alveolar macrophages develop and function at an O_2 tension of about 100 torr and exhibit relatively high levels of cytochrome oxidase activity and peritoneal macrophages develop and function at an O_2 tension of about 5 torr and exhibit relatively low levels of cytochrome oxidase activity (Simon et al, 1977); (3) the activities and contents of mitochondrial respiratory cytochromes increase after birth in rat liver, heart, and brain (Dawkins, 1959;

Hallman, 1971; Wilson, 1972; Pollak and Duck-Chong, 1973) and in dog heart (Mela et al, 1976), presumably in response to increased O_2 availability with the onset of pulmonary respiration.

To date, the only direct test of the possibility that changes in cytochrome oxidase activity accompany changes in O_2 availability *in vivo* has been our study of the effect of O_2 on the heart ventricles and brains of chick embryos maintained in 15% O_2 (hypoxic), 21% O_2 (ambient or normal), and 60% O_2 (hyperoxic) during the last 72h of incubation (Asson-Batres et al, 1989). We observed that cytochrome oxidase activity per mg DNA was 15-25% higher in hyperoxic ventricles than it was in hypoxic ventricles ($P < 0.05$). There was no effect of altered O_2 availability on the specific activity of cytochrome oxidase in embryonic brain ($P > 0.25$). We do not know the actual O_2 tensions of the tissues in these experiments; we can only say that an increase in incubator O_2 tension was associated with an increase in cytochrome oxidase activity in chick embryo heart ventricles, but not brain.

Although these studies support the notion that the effect of O_2 on the expression of respiratory cytochromes is of physiological significance *in vivo*, they are not definitive. Interpretation of the results is made difficult by the heterogeneous nature of tissue cell populations, the non-uniform distribution of tissue O_2 tensions, intermittent changes in cellular O_2 demands, and the complexity of the transition from intrauterine to independent life.

Thus, there are two very different and as yet, unanswered questions regarding the effect of O_2 on cytochrome oxidase activity in mammalian cells: (1) what is the mechanism for the effect of O_2 on the

expression of cytochrome oxidase in cells cultured under defined laboratory conditions of relatively "low" and "high" O_2 tensions (see Choice of Experimental and Control Oxygen Tensions below)? and (2) is the effect of O_2 on the expression cytochrome oxidase in cultured mammalian cells physiologically relevant to cells in the whole organism? I focused my thesis studies on the first question.

STATEMENT OF OBJECTIVES

The basis for the effect of O_2 on the expression of enzymes in the the mitochondrial electron transport chain of mammalian cells is unknown. It is possible that O_2 is acting at the level of gene transcription, mRNA translation, or assembly of the enzyme complexes. O_2 may coordinately affect the expession of several enzymes in the oxidative pathway, or, alternatively, the effect may be specific to heme-containing protein complexes. Since key enzyme complexes in the mitochondrial electron transport pathway are comprised of proteins that are made in the cytosol and in the mitochondrion, the effect of O_2 may be specific to nuclear or mitochondrial gene expression or to some aspect of protein import into the mitochondrion.

To investigate these possibilities, I examined the effect of O_2 on the expression of the cytochrome oxidase complex and the cytochrome b-c_1 complex in cultured mouse embryo fibroblasts. These enzymes were chosen because their levels of expression in mammalian cells have been shown to be affected by changes in O_2 availability and they are each composed of subunits coded for by nuclear and mitochondrial DNA.

DESCRIPTION OF THE ENZYMES CHOSEN FOR STUDY

Cytochrome *b-c*$_1$ (Ubiquinol-cytochrome c reductase) and cytochrome *c* oxidase are two of the four major enzyme complexes that make up the mitochondrial electron transport chain. In addition to facilitating the flow of electrons from NADH to molecular O_2, these complexes are sites at which protons are pumped across the inner mitochondrial membrane to create the proton gradient that is used to synthesize ATP.

Mammalian cytochrome *b-c*$_1$ is composed of 11 polypeptide subunits heme *b*, heme *c* , an iron-sulfur center and lipids. The cytochrome *b* subunit is synthesized in the mitochondria; the remaining subunits are translated in and imported from the cytoplasm (Wilson et al, 1981; Schagger et al, 1986; Gonzalez-Halphen et al, 1988).

Mammalian cytochrome oxidase is composed of 13 different polypeptides, heme *a*, heme *a* , two to three copper atoms, and lipids (Kadenbach et al, 1983; Steffens et al, 1987). The three largest polypeptides (subunits I, II, and III) are coded by mitochondrial DNA and translated on mitoribosomes (Anderson et al, 1981). The other polypeptides are coded by nuclear genes, translated on cytosolic ribosomes, and imported into the mitochondria (Hare et al, 1980).

CHOICE OF EXPERIMENTAL AND CONTROL OXYGEN TENSIONS

The results of many studies indicate the optimal level of O_2 for growth of cells *in vitro* is dependent on cell type and the parameter

defined as the benchmark of normal growth. Some cells, such as human fetal skin and lung fibroblasts, WI-38 fibroblasts, and fetal and newborn calf smooth muscle cells, proliferate equally well over a wide range of O_2 tensions (50-150 torr), although the population doubling time of WI-38 cells is slightly longer under room air conditions (O_2 tension, 134torr) (Balin et al, 1976; Webster and Burry, 1982; Benitz et al, 1986). Other cells, such as human and mouse hemopoietic stem cells and rat neonatal cerebral cortical neurons, survive and grow better at reduced O_2 tensions (48-68torr versus 135torr) (Bradley et al, 1978; Kaplan et al, 1986). A third group of cells, including human and mouse keratinocytes, monkey and porcine kidney cells, primary rabbit renal proximal tubules, primary rat heart and porcine pulmonary artery endothelial cells require high levels of O_2 (O_2 tensions, 120-158torr) to differentiate, to retain <u>in vivo</u> levels of oxidative metabolism, and to show optimal levels of cell proliferation (Acosta and Cheng-Pei, 1979; Taylor and Camalier, 1982; Pentland and Marcelo, 1983; Horikoshi et al, 1986; Block et al, 1989; Dickman and Mandel, 1989; Sahai et al, 1989). In contrast, (1), fibroblasts grown under relatively low O_2 tensions (20 and 75torr) synthesize less protein (as measured by the incorporation of [^{3}H] proline) than cells grown under an O_2 tension of 150torr (Webster and Burry, 1982; (2), WI-38 fibroblasts, kidney and foreskin cells, arterial smooth cells, and hemopoietic stem cells grow slower and have higher rates of glucose consumption and lactate production when grown under low O_2 tensions (<5-45torr) than when grown under higher O_2 tensions (26-144) (Balin et al, 1976; Taylor and Camalier, 1982; Pentland and Marcelo, 1983;

Horikoshi et al, 1986; Dickman and Mandel, 1989); and (3), heart and pulmonary artery cells exhibit signs of "hypoxic" injury when grown under O_2 tensions of <5-45 torr as compared with cells grown under O_2 tensions of 120-140torr (Acosta and Cheng-Pei, 1979; Block et al, 1989).

Thus, some cells proliferate best at O_2 tensions that are similar to those observed *in vivo*, some grow equally well over a wide range of O_2 tensions, and some differentiate and grow optimally at higher than physiologic O_2 tensions. Although the O_2 tension that promotes optimal growth is dependent on cell type, the evidence indicates that all cells do poorly when grown at very low O_2 tensions (<10 torr) (see references cited above).

It is not completely clear why, in some cases, O_2 tensions in excess of those measured *in vivo* support optimal cell growth and metabolism *in vitro*. The explanation likely has to do with the way O_2 is provided to cells (e.g., continuous flow-through system *in vivo* versus fixed-state systems or rocker-type systems *in vitro*), the amount of O_2 that is actually available to the cell (after diffusing from the transport system through the tissue or through the culture medium), and each cell's metabolic demands.

A relevant point is that what is normoxic for one cell may be hypoxic for another. Although some mammalian cells routinely experience *in vivo* O_2 tensions that are less than 40torr, it is likely that most adult mammals would experience clinical symptoms of hypoxia if the upper part of the range of available tissue O_2 tensions dropped

from 100torr to 40torr. Since there are more than 100 enzymes that require O_2 as a substrate in mammalian cells (Keevil and Mason, 1978) and since each has a different affinity for O_2, each cell's sensitivity to O_2 availability is likely to be at least partially dependent on the functional significance of particular oxygenases and oxidases to the cell's normal function and well being. Thus, although the K_{mO_2} for cytochrome oxidase function is accepted by many to be quite low (0.02-0.2μM in isolated mitochondria, Sugano et al, 1974) and although, in general, this enzyme utilizes more than 90% of the O_2 consumed by a cell, a functionally important oxidase or oxygenase with a relatively low overall requirement for O_2, but a relatively high K_{mO_2}, could well be a key determinant in events that define a cell's normoxic or hypoxic state. Dean Jones (1988) has summarized this phenomenon as follows: "Numerous enzymes are O_2 dependent in cells at normoxic O_2 concentrations; thus, hypoxia is a continuum with normoxia when one considers enzymatic O_2 dependence at the molecular level." Cell culture models have been developed and are currently being used to isolate and characterize the biochemical factors and pathways that contribute to or are responsible for the pathophysiological symptoms of hypoxia <u>in vivo</u> (Jones and Mason, 1978; Acosta and Cheng-Pei, 1979; Aw and Jones, 1982; Jones and Kennedy, 1982; Jones et al, 1983; Aw et al, 1987; Block et al, 1989).

O_2 Tensions Used in Published Studies of the Effect of O_2 on the Expression of Respiratory Cytochromes

Studies of the effect of O_2 on the respiratory cytochromes of yeast and bacteria (reviewed in Chapter One) have been carried out with cells grown in aerobiasis (room air) or anaerobiasis (achieved by either bubbling the cultures with ultrapure nitrogen (N_2) or by transferring them to Brewer jars made anaerobic with systems such as GasPaks (BBL Microbiology Systems). In the latter case, anaerobiasis was indicated by the presence of an indicator strip.

Studies of the effect of O_2 on mammalian cells have been carried out at what can be called "low" and "room air" O_2 conditions. Three stable, proliferating cell lines (strain L cells, human skin fibroblasts, L8 rat skeletal muscle cells), one transformed cell line (HeLa cells) and three primary cell types (hepatocytes, type II pneumocytes, and lung macrophages) show increased cytochrome oxidase activity when grown in 16-20% O_2 (relative to cells grown in cultures estimated or shown to have 0.3-3% residual O_2 in the cell culture medium) (Adebonojo et al, 1961; Hakami and Pious, 1967; Pious, 1970; Simon et al, 1977; Simon et al, 1978; Hare and Hodges, 1982; Murphy et al, 1984).

Hakami and Pious (1967; Pious, 1970) studied the effect of O_2 on a human skin fibroblast cell line. The "ambient-oxygen" cultures were flushed with 97% air,3% carbon dioxide (CO_2) and the "low-oxygen" cultures were alternately evacuated to 0.26atm and filled with 97% N_2, 3% CO_2 until an overflow valve set at slightly over 1 atm opened. After three cycles of suction and refilling, the residual O_2

concentration remaining in the glass flasks was estimated to be 0.3%. Cells were grown under these conditions for five days, harvested and analyzed for respiratory enzyme content. Cytochrome oxidase activities were three to seven times lower in cells grown under the low O_2 conditions than in cells grown under room air conditions (Hakami and Pious, 1967). The heme *a* +a_3, heme *b*, and heme *c* +c_1 difference spectra were barely detectable in the low $_2$ cells indicating a loss of functional respiratory cytochromes in these cells (Pious, 1970).

Hare and Hodges, (1982) measured cytochrome oxidase levels in rat liver hepatocytes immediately after isolation and after 24h exposure to either room air or low O_2 (99% N_2, 1% O_2). The cytochrome oxidase activity of freshly isolated cells was 60% less than that of similar cells cultured in room air for 24h (Hare and Hodges, 1982). Cellular heme *a* content (a measure of functional cytochrome oxidase content) was the same in hepatocytes exposed to low O_2 for 24h as it was in freshly isolated cells. In contrast, the heme *a* content of cells cultured under aerobic conditions doubled during the first 15h of culture. Hepatocytes normally experience *in vivo* O_2 tensions (5-40torr) that are significantly lower than the O_2 tension of humidified room air (150torr, STP). Thus, the experimental results indicate the increased level of O_2 available in humidified air leads to an increase in the amount of functional cytochrome oxidase present in freshly isolated hepatocytes. They also demonstrate the effect is not an artifact of cells cultured in room air for prolonged periods of time (i.e., human skin fibroblasts).

Investigators in Eugene Robin's laboratory (Simon et al, 1978; Murphy et al, 1984) refer to their cultures as "normoxic" (O_2 tension, 120-140torr) and "hypoxic" (O_2 tension, 15-25torr). In their studies, 96h growth under "hypoxia", resulted in cytochrome oxidase activities that were 55-57% of those present in cells grown under "normoxic" conditions for the same period of time. These results were obtained with a proliferating stable cell line (L8 rat skeletal muscle cells), non-proliferating primary cultures of mouse lung macrophages (obtained by lung mincing), and cloned type II pneumocytes isolated from rat lung. "Normoxic" culture conditions were achieved by gassing the flasks with 95% air,5% CO_2. "Hypoxia" was achieved by gassing flasks with 95% N_2, 5% CO_2. All cultures were regassed at 24 and 72h. Oxygen tensions of the cell culture medium were measured with a Corning blood gas analyzer and confirmed to be 120-140torr in the aerobic cultures and 15-25torr in the "hypoxic cultures over the 96h cultivation period.

Cell Culture Conditions Used For Experiments Reported in this Thesis

I used a stable cell line of AKR2B mouse embryo fibroblasts for the experiments reported in this thesis. I defined cell culture conditions that allowed me to address the question stated previously (p. 34-35): "what is the mechanism for the effect of O_2 on the expression of cytochrome oxidase in cells cultured under defined laboratory conditions of relatively "low" and "high" O_2 conditions?" Control cells gassed with 21% O_2, 5% CO_2, balance N_2 (procedures described in detail in Chapter Three, Experimental Methods) grew better

and expressed higher levels of cytochrome oxidase than experimental cells grown under the lowest O_2 tension I could produce by gassing flasks with 95% N_2, 5% CO_2. On average, after 24h of exposure, the low O_2 cells (average O_2 tension of the cell culture medium, 7torr) doubled once and exhibited about half as much cell-specific cytochrome oxidase activity as the control O_2 cells (average O_2 tension of the cell culture medium, 137torr). These results suggested that synthesis of new enzyme was inhibited in low O_2 and that pre-existing enzyme in the low O_2 cells was diluted out by the increase in cell number in the low O_2 cultures. I tested this hypothesis in the experiments reported in this thesis.

In preliminary experiments, I also grew HeLa (epithelial carcinoma), chinese hamster ovary (epithelial), NIH3T3 and L929 (mouse embryo embryo fibroblasts) under low and control O_2 conditions for 24h. In each case, the low O_2 cells had substantially less cytochrome oxidase activity than control cells. Hepatoma (epithelial tumor) cells did not survive under these conditions of low O_2 (data not shown).

CHAPTER THREE

EXPERIMENTAL METHODS

Cell culture: AKR2B cells, a continuous mouse embryo fibroblast cell line, were routinely cultured in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum and 10 units/ml penicillin-streptomycin (complete cell culture medium) in room air, 5% CO_2.

Procedures for maintaining and monitoring low and control O_2: Cells were passed to plastic T_{75} flasks containing 10ml complete cell culture medium (depth about 2mm). The flasks were capped with vented closures and maintained in a room air, 5% CO_2 incubator at 37°C until cell attachment (4-6 h). Subsequently, the vented caps were replaced with rubber sleeve stoppers and two 20 x 1" luer-lock syringe needles were inserted into each stopper. Each flask was equilibrated with a certified gas mixture delivered through tubing connected to the inlet syringe needle. The flasks were gassed with either 95% N_2, 5% CO_2 (low O_2 cultures) or 21% O_2, 5% CO_2, balance N_2 (control cultures) for 20 min on a gyratory shaker at room temperature in a tissue culture hood. After gassing, the needles were quickly removed and the stoppers self-sealed. As an added insurance against gas leaks, the low O_2 flasks were placed in an airtight incubator chamber box (O.D., 29.7 x 21.5 x 15.8cm; C.B.S. Scientific Co., Del Mar, CA) that was gassed for an additional 10 min with 95% N_2, 5% CO_2. The incubator chamber box

accommodated 12 T_{75} flasks and was maintained in a room air incubator at 37°C. Control cultures were maintained in a room air, 5% CO_2 incubator, or, in some experiments (to control for possible temperature effects), were placed in an incubator chamber box gassed with 21% O_2, 5% CO_2, balance N_2.

O_2 tension was monitored by analyzing medium samples with a Radiometer blood gas analyzer. Two-ml greased, glass syringes were rinsed with two back-to-back samples from a flask containing medium (but no cells) equilibrated with low or control O_2. The rinses were discarded and medium samples were withdrawn from the experimental cell culture flasks. The samples were stored on ice and analyzed within one-half hour of sampling. The O_2 tension of medium from at least two low and two control O_2 flasks was monitored at critical time points in each experiment.

Cell counts: Cells from at least two low and two control O_2 flasks were counted with a hemacytometer at critical time points in each experiment.

Enzyme activities: Cells from one flask were harvested with trypsin, washed once with phosphate buffered saline (PBS), and suspended in 0.25M sucrose, 10mM Tris-HCl, pH 7.4, 1mM EDTA. The cell suspension was sonicated for 30-50s. Cytochrome oxidase activity was measured spectrophotometrically at 37°C as described (Wharton and Tzagoloff, 1967) with the following modifications. The reaction mix contained 0.05M KPi, pH 7.0, 0.5% Tween-80, 10μM reduced cytochrome c, and a 0.1ml sample containing about 1×10^5 cells. The concentration and

reduction state of reduced cytochrome c was determined before each set of enzyme assays. Cytochrome c was greater than 90% reduced in all assays.

Succinate-cytochrome c reductase activity was determined as described (Tisdale, 1967).

Cellular O_2 Consumption Rates: The rate of O_2 consumption of whole cell suspensions was measured by following the O_2 content of cell suspensions over time. Approximately 2.6×10^7 fibroblasts, grown in either low or control O_2 for 48 h, were harvested with trypsin, washed once with PBS, and mixed with a suspension of washed sheep red blood cells in serum-deprived cell culture medium (5 ml final fluid volume, Hct = 16). The red blood cells were used to provide a source of O_2 for the fibroblasts. After the suspension was swirled in room air to oxygenate the red blood cells, it was withdrawn into a greased glass syringe and incubated at 37°C. Five aliquots from a sample were injected, one after the other, into a Lex-O_2-Con O_2 content analyzer over a 40min period. The rate of O_2 consumption of a blank (red blood cells only) was also determined. The volume % O_2 (ml O_2 consumed per 100 ml cell suspension) was plotted against time, and the slope of the best fit line determined by least squares regression was used to calculate the rate of O_2 consumption.

Labeling and protein translation inhibitor studies: Proteins in low and control O_2 cells were labeled for 1-2h with 5-40 μCi/ml [^{35}S] methionine (New England Nuclear cell label grade, > 660 Ci/mmol) in

media minus methionine, supplemented with 5% dialyzed FBS + 8mM glutamine. To specifically label the mitochondrial translation products, the cells were pre-incubated (20min) and labeled in the presence of 0.2 mg/ml cycloheximide. In some studies, the labeling medium was removed, the cells were rinsed 3 x with PBS, equilibrated with low or control O_2, and chased for varying lengths of time in 10ml complete cell culture medium supplemented with 2mM methionine.

The incorporation of [^{3}H] thymidine was measured in low and control O_2 cells by giving them a 2h pulse of 0.5-1 μCi/ml [^{3}H] thymidine (New England Nuclear, 20 Ci/mmol). Cells were either harvested immediately after the pulse or chased for 24h in the absence of the radioactive precursor.

Mitochondrial Preparation: Cells were harvested with trypsin, washed with isotonic buffer, swelled with hypotonic buffer, and disrupted by homogenization as described (Attardi et al, 1969). The homogenate was brought to 0.25M sucrose and centrifuged at 1160g for 3min to remove nuclei and cell debris. The supernate was centrifuged in a Sorvall SS-34 rotor at 11950g for 30min to pellet the mitochondria. The pellet was lysed with PBS buffer brought to 1% Triton X-100, 0.5μM phenylmethylsulfonyl fluoride (PMSF), 10mM iodoacetamide, 1μM 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK), and 1 g/ml of each: pepstatin, antipain, leupeptin. The lysate was centrifuged 5min to remove insoluble protein and the supernate was precipitated with 10% TCA. The precipitate was washed with 10% 0.1M HCl, 90% acetone and pelleted with a microcentrifuge. The pellet was dried in room air and

dissolved in electrophoresis sample buffer (Maizel, 1969).

Preparation of membrane pellets: Cells were harvested with trypsin, washed once with complete cell culture medium, once with PBS, and suspended in 1ml 0.25M sucrose, 10mM Tris-HCl, pH 7.4, 1mM EDTA. Cell suspensions were sonicated for 2-3min, transferred to 2ml nitrocellulose tubes, and centrifuged in a Beckman TI50 rotor at 106,000g for 1h at 4° C.

Mitochondrial Translation Products: Cell membrane pellets containing labeled mitochondrial translation products were sonicated for 30-50s, heated at 95-100°C for 5min, cooled, and brought to 1% 2-mercaptoethanol. Some mitochondrial translation product samples were heated at 37°C for 1h; electrophoretic separations of these samples were identical to those heated at the higher temperature.

Purified Enzymes: Cytochrome b-c_1 was purified from beef heart mitochondria as described (Riccio et al, 1977). A sample of purified beef heart cytochrome oxidase was generously provided by Dr. N. Kennaway (Oregon Health Sciences University) and Dr. R.A. Capaldi (University of Oregon).

Antiserum: Dr. J.F. Hare (Oregon Health Science University) kindly provided antisera against holocytochrome c oxidase and against holocytochrome b-c_1 for these studies. The antisera were raised in rabbits as described (Wilson et al, 1981). The subunit specificity of each antiserum was checked by immunoblotting (Burnette, 1981).

Immunoprecipitation: Procedures for immunoprecipitating cytochrome oxidase were carried out on ice or at 4°C. Cell membrane pellets were lysed with ice-cold 2% Triton X-100, 0.05M Tris-HCl, pH 7.4, 1mM PMSF, sonicated briefly, and centrifuged in a TI50 rotor at 106,000g for 45min at 4°C to remove insoluble protein. Lysates were rotated end-over-end with preimmune antisera for 1h and with 50µl fixed Staphylococcus aureus (Calbiochem) for 30min. Antibody conjugates were pelleted with a microcentrifuge and 50µl anti-cytochrome oxidase antisera and 1 mg/ml bovine serum albumin were added to the lysates. The reaction mixtures were rotated end-over end for 20h, mixed with 50µl fixed S. aureus for 30min, and spun in a microcentrifuge for 2min. The pellets were washed once with lysis buffer, once with PBS diluted with 10 volumes of distilled water, and again with lysis buffer. 50 µl anti-cytochrome oxidase antisera failed to remove more enzyme from post-immunoprecipitate supernates. Immunoprecipitates were dissolved in SDS sample buffer (see Electrophoresis) and heated at 95°C for 5min or 40°C for 30min.

Membranes prepared from cells labeled in the absence of cycloheximide were immunoprecipitated with 50µl cytochrome b-c_1 antisera as described for cytochrome oxidase. Cell membranes from cells labeled in the presence of inhibitor were lysed with 2% Triton X-100, 10mM MOPS, pH 7.4, 0.1M NaCl, 1mM EDTA, 5mM antimycin A, 1mM PMSF, and immunoprecipitated with cytochrome b-c_1 as described for cytochrome oxidase with the exception that the immunoprecipitates were washed once with 2% Triton X-100, 10mM NaPi, pH 7.4, 0.1 mM NaCl, 1mM EDTA, 1 mg/ml bovine serum albumin, 1mM PMSF, twice with the above buffer, but with

0.5M NaCl and 0.05% Triton X-100, and twice with buffer adjusted to 0.05% TX-100. Immunoprecipitates were dissociated in SDS sample buffer (see Electrophoresis) and heated at 37°C for 2h or 50°C for 20min.

Analysis of Postmembrane and Post-Immunoprecipitate Supernates: Labeled mitochondrial translation products in postmembrane supernates were precipitated with 10% trichloroacetic acid (TCA), washed with 10% (0.1N) HCl, 90% acetone, and dissolved in SDS sample buffer (see Electrophoresis). Alternatively, cell membrane lysates from cells labeled in the presence of cycloheximide were reacted with cytochrome oxidase antisera in the absence of bovine serum albumin, and, after removal of Triton X-100 on Biobead SM2 (Rivett and Hare, 1987), the post-immunoprecipitate supernates were dialyzed (in Spectra, Por 1 dialysis tubing) for 24h against three changes of distilled water. Dialyzed samples were dried in a vacuum concentrator for 3h, and dissolved in electrophoresis sample buffer (see below).

Electrophoresis: Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed in slab gels according to two different published methods (Maizel, 1969; Schägger and von Jagow, 1987). Samples were dissociated in sample buffers (with or without reducing agents) appropriate for each procedure. Gels were dried, fluorographed with Enhance™(New England Nuclear) or Fluoro-hance™ (Research Products International, Corp.) as described in the product literature, and exposed to Kodak X-OMAT film at -70°C.

Two dimensional polyacrylamide gel electrophoresis was carried out

according to the methods of O'Farrell (1975) and Baumann and Doyle (1979).

Determination of Radioactive Counts in Cell Fractions: [^{35}S] methionine-labeled proteins in whole cell suspensions or post-membrane supernates were precipitated with TCA and washed with acid/acetone as described above. Washed pellets were solubilized in a minimal amount of 0.1N NaOH, heated to 56°C for 10min, diluted as necessary with distilled water, and counted in toluene based scintillation cocktail.

Determination of Radioactive Counts in Gel Slices: Corresponding fluorographs were used to locate polypeptide bands of interest on dried polyacrylamide gels. The bands were excised, mixed with 0.2ml 60% perchloric acid plus 0.4ml fresh 30% hydrogen peroxide in tightly capped 22ml glass vials, and incubated in a 60°C shaking water bath until completely dissolved (about 20h). Ten ml toluene based scintillation cocktail were added, and they were counted after 1h.

Densitometry. Fluorographs were scanned with a Bio-Rad Model 620 video densitometer and the areas under specified peaks were integrated with a computer assisted software program.

Comparison of Densitometry with Radioactive Counts in Gel Slices: The accuracy of the densitometric method of analysis was evaluated in two ways: (1), I compared the maximal optical densities (O.D.) of protein band images on fluorographs with the corresponding radioactivities of protein bands cut from dried gels. This analysis was necessary in order to define the range of meaningful O.D. values; in particular, I

wanted to know if there was a loss of correspondence between maximal optical density (a measure of film image intensity) and the radioactivity of corresponding protein bands for film images that were either very faint or very intense; (2), I compared the integrated areas under specified protein peaks on densitometric scans with the known radioactivity of each protein band. The area under a given peak is a more accurate representation of the quantity of a protein than is the maximal optical density of its photographic image on the fluorograph because proteins often run as diffuse bands on a gel.

There was a linear correlation between the peak absorbances of protein bands on fluorographs and the total radioactivities of corresponding bands removed from the gels over an O.D. range of 0.09-1.46 O.D. units (Fig. 1). Thus, in subsequent analyses, I included only those data from control cultures that fell within this absorbance range. The expression of some of the proteins under study was affected by a decrease in O_2 availability to the cells. As a consequence, the absorbances of affected proteins from low O_2 cells were less than those of the absorbances of the corresponding proteins from control O_2 cells. Even so, with the exception of two data points, all data from low O_2 cultures also fell within the acceptable O.D. range.

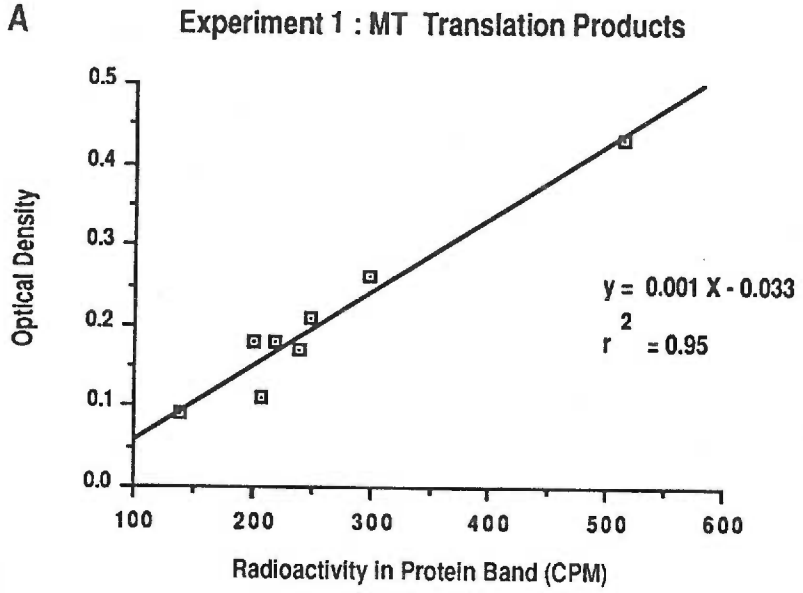
A direct relationship between the integrated areas under specified protein peaks on densitometric scans and known radioactivities of each protein band is shown in Figs. 2 and 3. Comparisons were made for all gel separations where both types of analysis were available (Figs. 3-4). The equation of the best fit curve for each comparison was

Fig. 1. Correlation between the radioactivity in a sample and the maximal absorbance (optical density) of its fluorographic film image. Samples are mitochondrial (MT) translation products or cytochrome oxidase (COX) immunoprecipitates prepared from low and control oxygen cells as described (see under appropriate sections of Experimental Methods above and Chapter Four). Samples were separated on SDS polyacrylamide gels. The amount of radioactivity in gel slices was determined as described above (see Determination of Radioactive Counts in Gel Slice). Fluorographs were prepared from dried gels and scanned with a densitometer. The equation for the best fit line was determined by least squares regression and the coefficient of determination (r^2) was calculated. All correlations are highly significant ($P < 0.01$).

A. Mitochondrial translation products from low and control oxygen cells labeled in the presence of cycloheximide; this experiment corresponds to experiment 1 in Table II, Chapter Five, p. 75.

B. Cytochrome oxidase immunoprecipitates from low and control oxygen cells labeled in the presence and absence of cycloheximide (all samples run on the same gel); this experiment corresponds to experiment 2 (+ CHI, - CHI) in Table I, Chapter Five, p. 74.

A


Experiment 1 : MT Translation Products
Optical Density
0.5
0.4
0.3
0.2
0.1
0.0
100
200
300
400
500
600
Radioactivity in Protein Band (CPM)
y = 0.001 X - 0.033
r 2 = 0.95

B

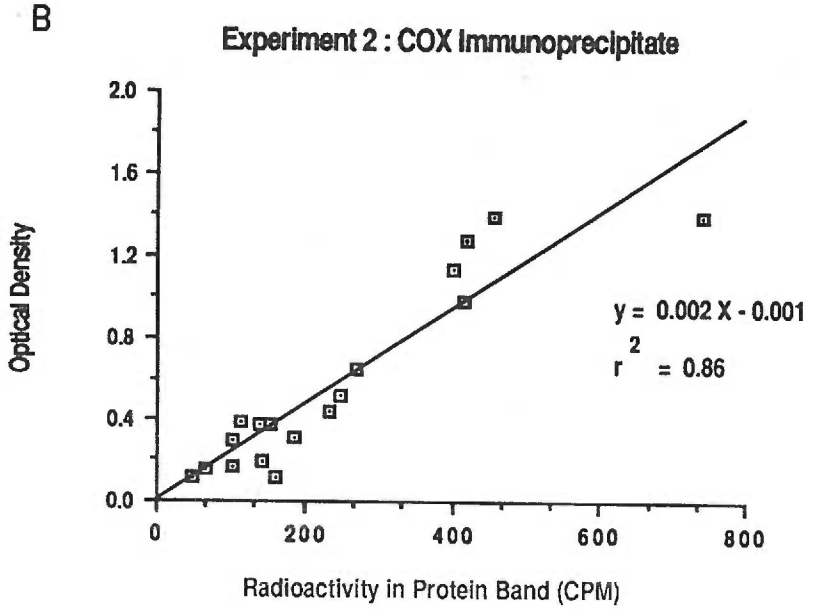

Experiment 2 : COX Immunoprecipitate
Optical Density
2.0
1.6
1.2
0.8
0.4
0.0
0
200
400
600
800
Radioactivity in Protein Band (CPM)
y = 0.002 X - 0.001
r 2 = 0.86

Fig. 1, continued.

C. Cytochrome oxidase immunoprecipitates from low and control oxygen cells labeled in the presence of cycloheximide (+CHI); this experiment corresponds to experiment 3 in Table I (+ CHI), Chapter Five, p. 74.

C

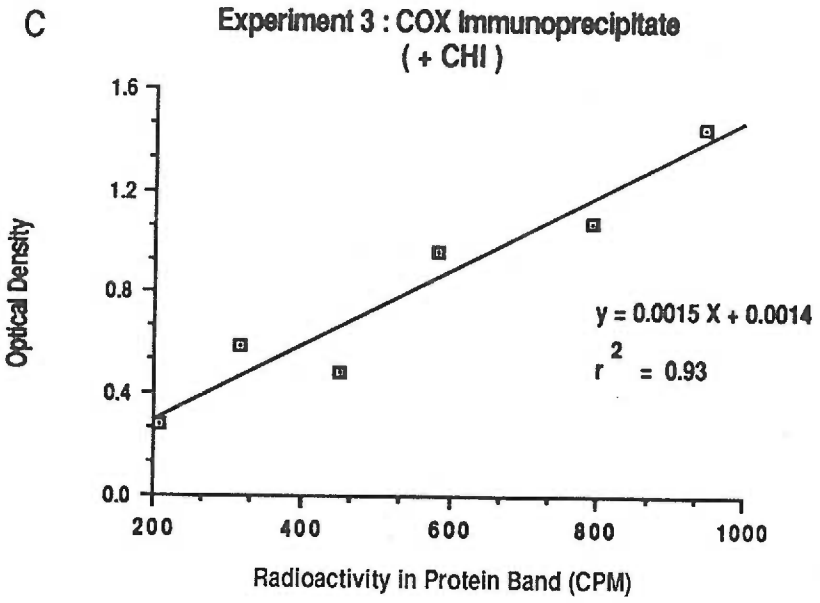

Experiment 3 : COX Immunoprecipitate
(+ CHI)
Optical Density
1.6
1.2
0.8
0.4
0.0
200
400
600
800
1000
y = 0.0015 X + 0.0014
r 2 = 0.93
Radioactivity in Protein Band (CPM)

Fig. 2. Correlation between the radioactivity in a protein band and the absorbance of its fluorographic film image (integrated area under the curve, mm^2). Samples are cytochrome oxidase immunoprecipitates prepared from low and control oxygen cells. Experiments 2 and 3 are those reported in Table I, Chapter Five, p. 74). Samples labeled in the presence and absence of cycloheximide were run on the same gel in experiment 2. Samples labeled in the presence (+) and absence (-) of cycloheximide (CHI) were run on separate gels in experiment 3. Radioactivities and densitometric scans were determined as described in the legend to Fig. 1. The areas under curves of interest were integrated with a computer assisted software program. All correlations are highly significant ($P < 0.01$).

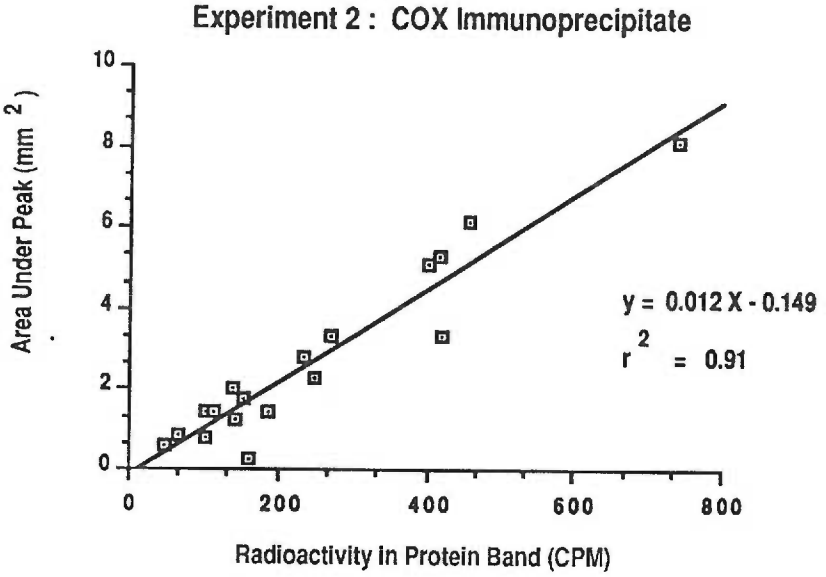
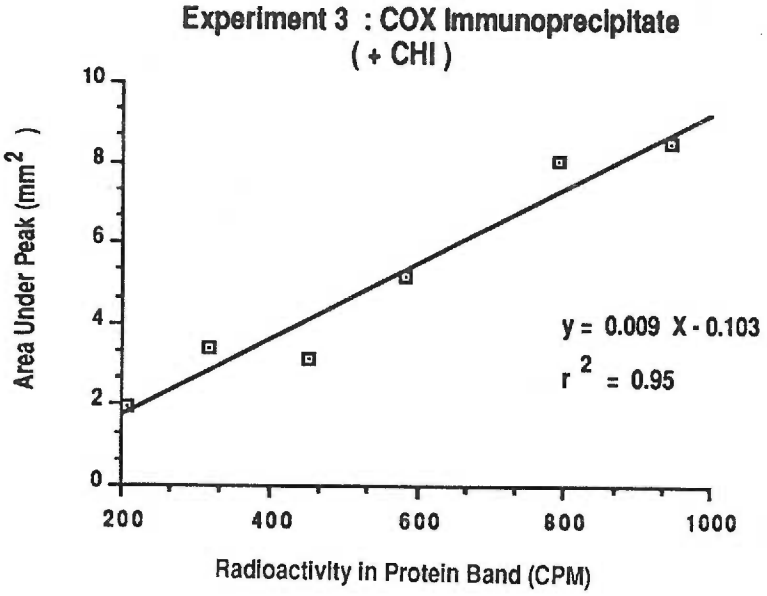

Experiment 2 : COX Immunoprecipitate
Area Under Peak (mm 2)
10
8
6
4
2
0
0
200
400
600
800
Radioactivity in Protein Band (CPM)
y = 0.012 X - 0.149
r 2 = 0.91

Fig. 2, continued.


Experiment 3 : COX Immunoprecipitate
(+ CHI)
Area Under Peak (mm2)
10
8
6
4
2
0
200
400
600
800
1000
Radioactivity in Protein Band (CPM)
y = 0.009 X - 0.103
r 2 = 0.95

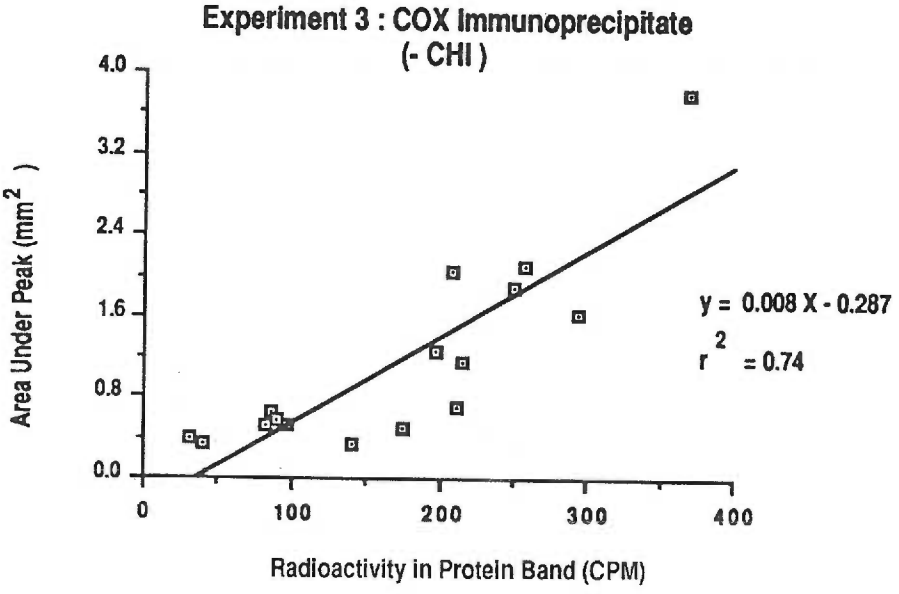
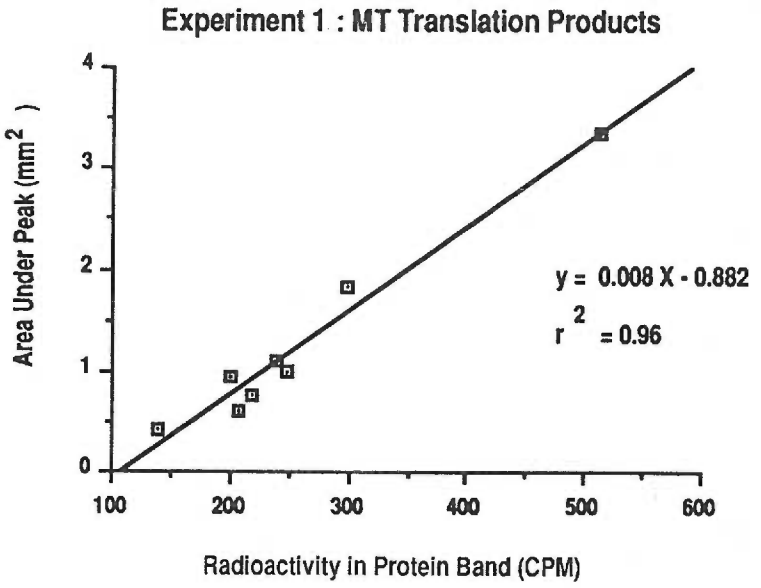

Experiment 3 : COX Immunoprecipitate
(- CHI)
Area Under Peak (mm2)
4.0
3.2
2.4
1.6
0.8
0.0
0
100
200
300
400
Radioactivity in Protein Band (CPM)
y = 0.008 X - 0.287
r 2 = 0.74

Fig. 3. Correlation between the radioactivity in a protein band and the absorbance of its fluorographic film image (integrated area under the curve). Samples are mitochondrial (MT) translation products prepared from low and control oxygen cells. Experiments 1, 3, and 4 correspond to those reported in Table II, Chapter Five, p. 75). Samples were run on SDS polyacrylamide gels and analyzed as described in the legend to Fig. 1. All correlations are highly significant ($P < 0.01$).


Experiment 1 : MT Translation Products
Area Under Peak (mm2)
Radioactivity in Protein Band (CPM)
y = 0.008 X - 0.882
r 2 = 0.96
0
1
2
3
4
100
200
300
400
500
600

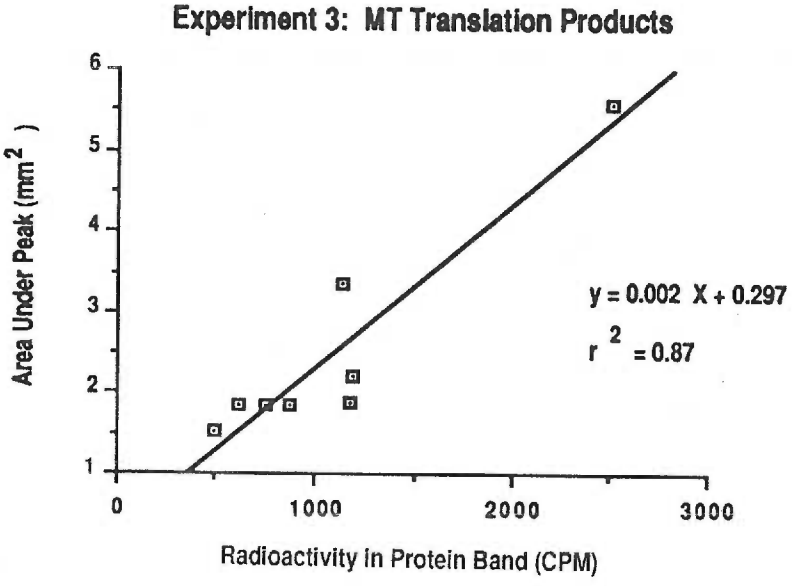
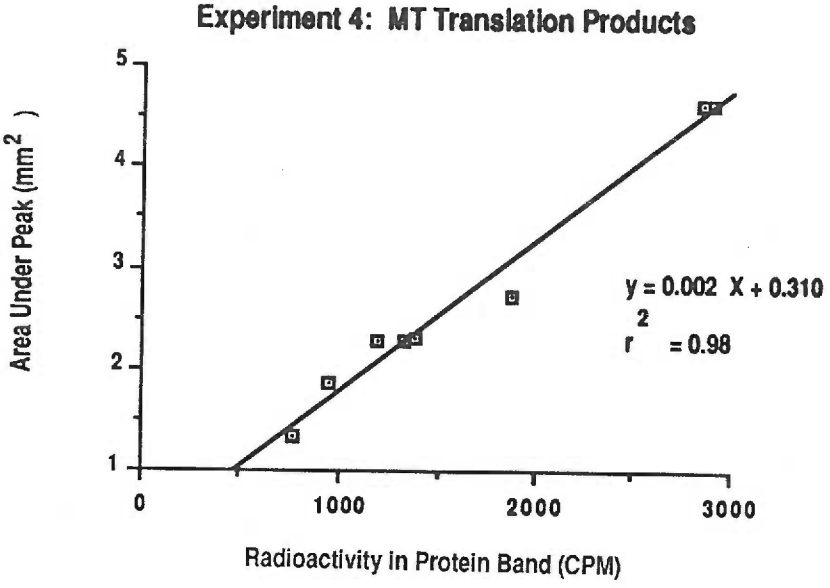

Experiment 3: MT Translation Products
Area Under Peak (mm2)
Radioactivity in Protein Band (CPM)
y = 0.002 X + 0.297
r 2 = 0.87
1
2
3
4
5
6
0
1000
2000
3000

Fig. 3, continued.


Experiment 4: MT Translation Products
Area Under Peak (mm2)
5
4
3
2
1
0
1000
2000
3000
y = 0.002 X + 0.310
r2 = 0.98
Radioactivity in Protein Band (CPM)

determined by least squares regression and the coefficient of determination (r^2) was calculated to statistically test the linearity between data obtained from the two methods of analysis. There was a good fit for all of the analyzed data (r^2 range from 0.74-0.98), and the correlations were highly significant ($P < 0.01$). There are differences in the slopes and Y-intercepts of curves fit to the various data sets because intensities of the film image vary as a function of sample radioactivity and the film exposure time.

The conclusion to be drawn from these analyses is that the data obtained from the densitometric method of analysis are comparable to those obtained from direct determinations of the radioactivities of individual protein bands. There was a positive correlation between the peak absorbances of fluorographic images and the radioactivities of the corresponding protein bands over an O.D. range of 0.09-1.46 O.D. Units. Areas under peaks of interest also correlated with the radioactivities of corresponding gel slices, and are used to quantitate the amounts of specified proteins in low and control O_2 samples.

Other Methods. Cytochrome difference spectra were determined on whole cell suspensions and mitochondrial preparations by a published method (Williams, 1964).

CHAPTER FOUR

EFFECT OF OXYGEN ON CELL GROWTH, PROTEIN AND DNA SYNTHESIS, AND THE ACTIVITIES OF CYTOCHROME OXIDASE AND SUCCINATE-CYTOCHROME c REDUCTASE

Cell Culture Conditions

The procedures for establishing and maintaining low and control O_2 tensions in cell culture media were effective and reproducible over a 48h period (Table I). For consistency with other data summarized in Table I, the average O_2 tension of only 4 experiments are reported; however, measurements made in many experiments confirm the reproducibility of these values (Table II). The measured O_2 tensions, correspond to O_2 concentrations of 11 ± 6μM (low O_2 cultures, 24h); 10 ± 5μM (low O_2 cultures, 48h); 229 ± 16μM (control O_2, 24h) and 226 ± 22μM (control O_2, 48h).

Effect of O_2 on Cell Morphology

The morphology of AKR2B cells grown in low O_2 for 48h was indistinguishable from that of control cells (Fig. 1).

Effect of O_2 on Cell Loss

Cell detachment was negligible in both the low and control O_2 cultures over a 48h period. In addition, the amount of radioactivity remaining in low and control O_2 cultures pre-labeled with [^{3}H] thymidine was similar after 24h. The radioactivity present in cells pre-labeled with [^{3}H] thymidine and chased in label free medium for 24h

Table I. Comparison of cell culture conditions, cell proliferation, DNA synthesis, cell loss and enzyme activities after 24 and 48h growth in low or control oxygen. Individual T_{75} (80 cm^2) flasks were plated with ca. 8×10^5 cells each and were maintained in a room air/5% carbon dioxide incubator for 6-7h to allow for cell attachment. Subsequently, an equal number of flasks were gassed and maintained in either low or control oxygen for 24 or 48h. Oxygen tension of the cell culture medium, cell number and enzyme activity were determined as described under Experimental Methods (Chapter 3). In some experiments, cells were labeled with [^{3}H] thymidine for 2h in room air and chased for 24 or 48h in low or control oxygen. In other experiments, they were pulsed with [^{3}H] thymidine for 2h at the 24 or 48h time points and harvested immediately after the pulse. The average $\pm$ (SD) of data from several experiments or of data from multiple flasks analyzed in a single experiment are reported. The number of flasks analyzed per treatment in each experiment is indicated. SD are not reported for data from fewer than three flasks.

Variable	No. Expts. (No. flasks/treatment in each expt.)	Treatment	24h X ± (SD)	48h X ± (SD)
Oxygen Tension (torr)	4 (2)	Low O_2	7 (4)	6 (3)
		Control O_2	143 (10)	141 (14)
Cell Number[1] (x 10^6)	4 (2)	Low O_2	1.4 (0.4)	1.4 (0.7)
		Control O_2	1.7 (0.4)	4.2 (1.2)
		TCA Precipitable Counts (CPM/10^6 Cells)		
[^{3}H] thymidine (2h pulse after 24h or 48h growth)	1 (3)	Low O_2	4150 (1679)	5743 (1508)
		Control O_2	4119 (579)	1879 (1230)
[^{3}H] thymidine[2] (Labeled before 24h or 48h chase)	1 (2)	Low O_2	804	626
		Control O_2	907	803
		Enzyme Activities		
Cytochrome Oxidase[3]	4 (2)	Low O_2	35 (9)	19 (10)
		Control O_2	67 (12)	65 (18)
Succinate-Cytochrome c Reductase[4]	1 (2)	Low O_2	15.2 (1.9)	10.2 (0.7)
		Control O_2	20.1 (3.0)	16.0 (2.5)

[1] 0.6 (± 0.01) cells at 0h time point for low and control O_2 cultures.

[2] CPM/10^6 cells = 1045 for samples harvested immediately after 2h label in room air

[3] nmol substrate ox/min/10^6 cells

[4] nmol substrate red/min/10^6 cells

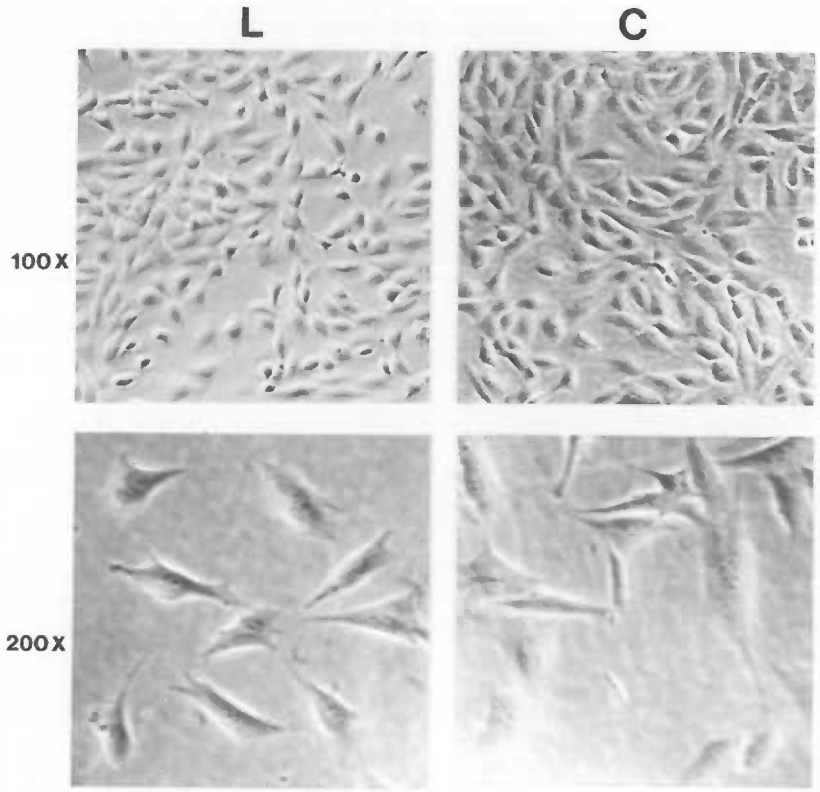
Table II. Comparison of cell culture conditions, cell proliferation, and cytochrome oxidase activity after 24h growth in low and control oxygen. Individual T_{75} (80 cm^2) flasks were plated with ca. 8×10^5 cells each and were maintained in a room air/5% carbon dioxide incubator for 6-7h to allow for cell attachment. Subsequently, an equal number of flasks were gassed and maintained in either low or control oxygen for 24h. Oxygen tension of the cell culture medium, cell number and enzyme activity were determined as described under Experimental Methods, Chapter Three. The average ± (SD) of data from each of two flasks per treatment in 12 different experiments is reported. Data from low and control oxygen sample groups were compared with a paired t-test.

Variable	Number of Experiments (No. flasks/treatment in each expt.)	Control Oxygen	Low Oxygen	% of Control
Oxygen Tension (torr)	12 (2)	137 (± 24)	7 (± 4)	
Cell Number ($\times 10^6$)	12 (2)	2.0 (± 0.4)	1.4* (± 0.3)	73 (± 15)
Cytochrome Oxidase Activity[1]	12 (2)	68 (± 12)	38* (± 12)	55 (± 9)

[1] nmol substrate ox/min/10^6 cells

*$P < 0.05$, relative to control O_2 value.

Fig. 1. Cell morphology after 48h exposure to low (L) or control (C) oxygen. Attached AKR2B fibroblasts were photographed with a 35mm camera through an inverted phase contrast microscope at 100X and 200X magnification.


L
C
100X
200X

was 77% (in low O_2 cells) and 87% (in control O_2 cells) of that present in cells labeled in room air and harvested immediately after the pulse (Table I). Since DNA isn't degraded, the radioactivity remaining in pre-labeled cultures provides an estimate of cell survival. After a 48h chase, specific radioactivity remaining in the low O_2 cultures was 78% of that remaining in control O_2 cultures chased for the same amount of time. (Table I).

Effect of O_2 on Cell Proliferation

DNA synthesis continued for 48h in both low and control O_2 cells (Table I). However, the specific radioactivity of control O_2 cells pulse-labeled at the 48h time point was substantially lower than that of control O_2 cells pulse-labeled at the 24h time point. Presumably, this was because the 48h control O_2 cultures were reaching confluence (Table I).

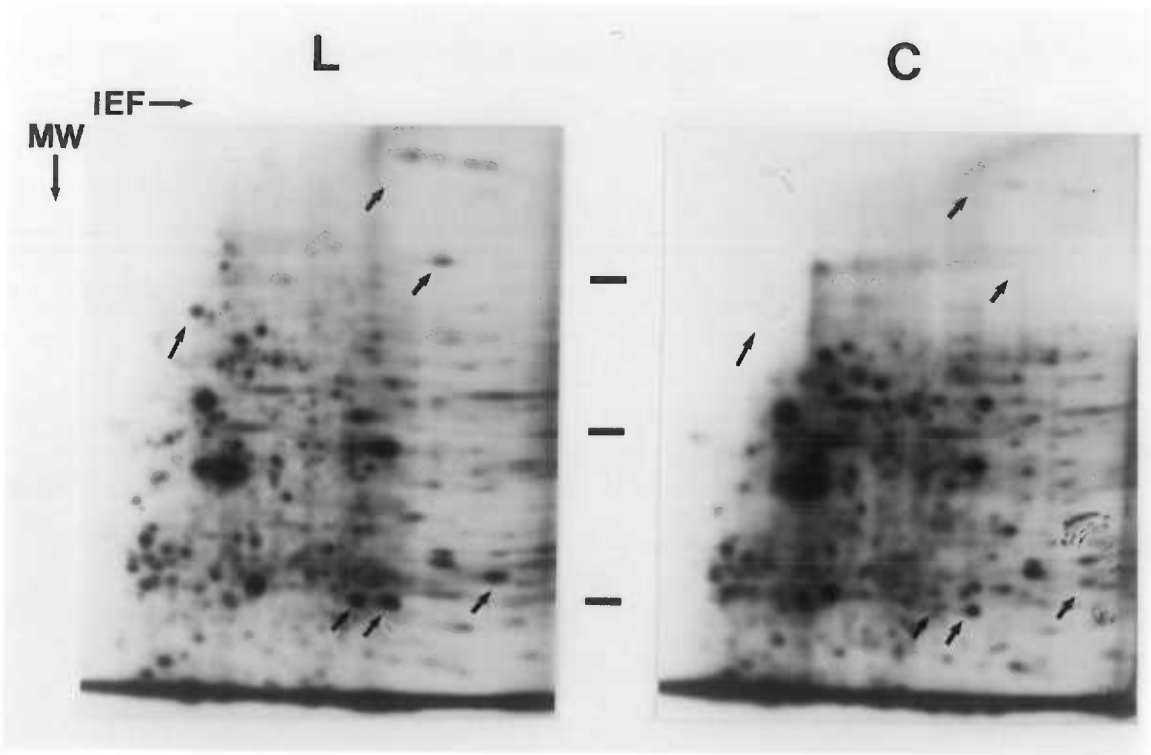
Control O_2 cells seeded at 0.8×10^6 cells/T_{75} flask proliferated logarithmically throughout 48h of growth and reached average densities of 4.2×10^6 cells/flask (Table I). Low O_2 cells doubled once in 24h and showed no net increase in cell number/flask during the second day of growth (Table I). Maintainance of the same average number of cells between 24h and 48h in the low O_2 cultures required a low level of continued proliferation since some cell loss occurred during this time period (see above). The densities of the low O_2 cultures were significantly less than those of the control O_2 cultures after 24h growth (Table II). After 72h of growth, low O_2 cultures were still sub-confluent (data not shown).

Effect of O_2 on Protein Synthesis

Protein synthesis continued in the low O_2 cultures, however the relative amount of newly synthesized protein in the low O_2 cultures varied from experiment to experiment. Cell-specific TCA precipitable [^{35}S] methionine counts in the low O_2 whole cell lysate were 60% of control for the experiment shown in Fig. 2. In five other experiments, cell-specific counts present in whole cell lysates or the soluble protein fraction (postmembrane supernate) from low O_2 cells ranged from 61 to 98% of control (see discussion, Chapter Five, p. 77). The reason for the variability is not known. Cells were exposed to low O_2 for the same amount of time in each experiment.

The general pattern of proteins synthesized by cells exposed to low O_2 for 24h was similar to that of control cells although there appeared to be increased synthesis of at least 8 unidentified proteins in low O_2 cells (Fig. 2). An induction of anoxic stress proteins or oxygen regulated proteins (ORPs) has been observed in EMT6/Ro cells, chinese hamster ovary cells, rat fibroblasts, maize primary roots, and Escherichia coli grown under extreme hypoxia or anaerobiasis (Sachs et al, 1980; Smith and Neidhardt 1983; Sciandra et al, 1984; Heacock and Sutherland, 1986; Anderson et al, 1989). Several of the ORPs have been identified and include cathepsin L (Anderson et al, 1989) and proteins involved in anaerobic energy metabolism (Sciandra et al, 1984; Winkelman and Clark, 1986 and refs. therein; Dennis et al, 1988 and refs. therein). Regulation of the expression of these proteins occurs at the level of gene transcription. The role of oxygen in the process

Fig. 2. Comparison of the pattern of protein synthesis in AKR2B fibroblasts grown for 24h in low (L) and control (C) oxygen. Twenty-four hours after gassing, the cells were pulsed with [^{35}S] methionine for 2h in the presence of low and control oxygen and harvested immediately. The cell pellets were lysed with SDS sample buffer and separated by isoelectric point in the first dimension and molecular weight in the second dimension. The low oxygen gel was loaded with an aliquot of cell lysate equivalent to 1.6×10^4 cells; the control oxygen aliquot was equivalent to 1.3×10^4 cells. Several proteins (indicated by the arrows) appear to have been induced in cells grown in low oxygen.


L
C
IEF
MW

is not understood, but one proposal is that a gene regulatory element binds a protein that undergoes a conformational change following exposure of the cells to anaerobic growth conditions (Winkelman and Clark, 1986).

Effect of O_2 on Enzyme Activities

Cells grown under low O_2 showed a consistent decrease in cytochrome oxidase activity over time relative to control cells. Cell specific activity of the low O_2 cells was 52-55% of control after 24h (Tables I and II) and 29% of control after 48h (Table I). To a lesser degree, succinate-cytochrome c reductase activity (a measure of the activity of the cytochrome b-c_1 complex) also declined in cells grown in low O_2 for 48h. Relative to controls, cell specific enzyme activity of low O_2 cells was decreased by 24% after 24h and 36% after 48h.

The O_2 consumption rate of cells grown in low O_2 for 48h was 42% of control (low O_2 cells: 0.075 µmol O_2 per hour per 10^6 cells; control O_2 cells: 0.179 µmol O_2 per hour per 10^6 cells). In this experiment, cell-specific cytochrome oxidase activity in the low O_2 cells was 52% of control.

I was unsuccessful in obtaining cytochrome difference spectra from either AKR2B cell suspensions or isolated mitochondria, and thus, I was unable to estimate the contents of hemes a + a_1, b, and c + c_1 in the low and control O_2 cells. It may be that AKR2B cells contain cell-specific chromogens that interfere with the spectrophotometric detection of cytochrome hemes as other cell types at similar cell concentrations exhibit clear difference spectra.

Reversibility of the Effect of O_2 on Cell Proliferation and Cytochrome Oxidase Activity

The effect of O_2 on cell proliferation and cytochrome oxidase activity was reversible within 24h of exposure to control O_2 (Table III). The restoration of cytochrome oxidase activity was not detectable within 2h, suggesting the defect was not merely due to an O_2-induced conformational change in the state of the complex. A time consuming process, such as translation, assembly, or import of one of the components of the complex, was required to restore enzyme function.

Effect of O_2 on AKR2B Cell Growth and the Design of Further Experiments

Subsequent experiments reported in this thesis were designed with an awareness of the diverse effects that decreased O_2 availability has on cell growth and metabolism. My tests of the effects of O_2 on the synthesis and assembly of cytochrome oxidase and cytochrome b-c_1 were carried out on cells that were grown in low O_2 for 24h. Cells exposed to low O_2 for this length of time were viable (as judged by their morphology, survival, and ability to proliferate and synthesize DNA and protein) and yet, exhibited a sufficient reduction in cytochrome oxidase activity to allow characterization of the response.

Table III. Reversibility of the effect of oxygen on cell proliferation and cytochrome oxidase activity. Flasks were plated with 8×10^5 cells/flask. After cell attachment in room air, some flasks were harvested immediately (0h), some were grown in low or control oxygen for 24h or 48h, and some were grown in low oxygen for 48h and then switched to control oxygen for an additional 2h (+ 2h) or 24h (+ 24h). Data are the average ± (SD) of cells from 3 flasks, except where indicated.

Time After Gassing	Cell Number[1]		Cytochrome Oxidase Activity[2]	
	Low O_2	Control O_2	Low O_2	Control O_2
0h	0.80 (±0.05)	0.80 (±0.05)	73@	73@
24h	1.3 (±0.1)	1.8 (±0.2)	41 (± 5)	62 (±14)
48h	2.0 (±0.2)	5.9 (±0.2)	25 (± 4)	59 (± 7)
+2h	2.6 (±0.5)		25 (± 4)	
+24h	4.9 (±0.5)		66 (±14)	

[1]No. Cells x 10^6

[2]nmol substrate ox/min/10^6 cells

@the average activity of cells from two flasks

CHAPTER FIVE

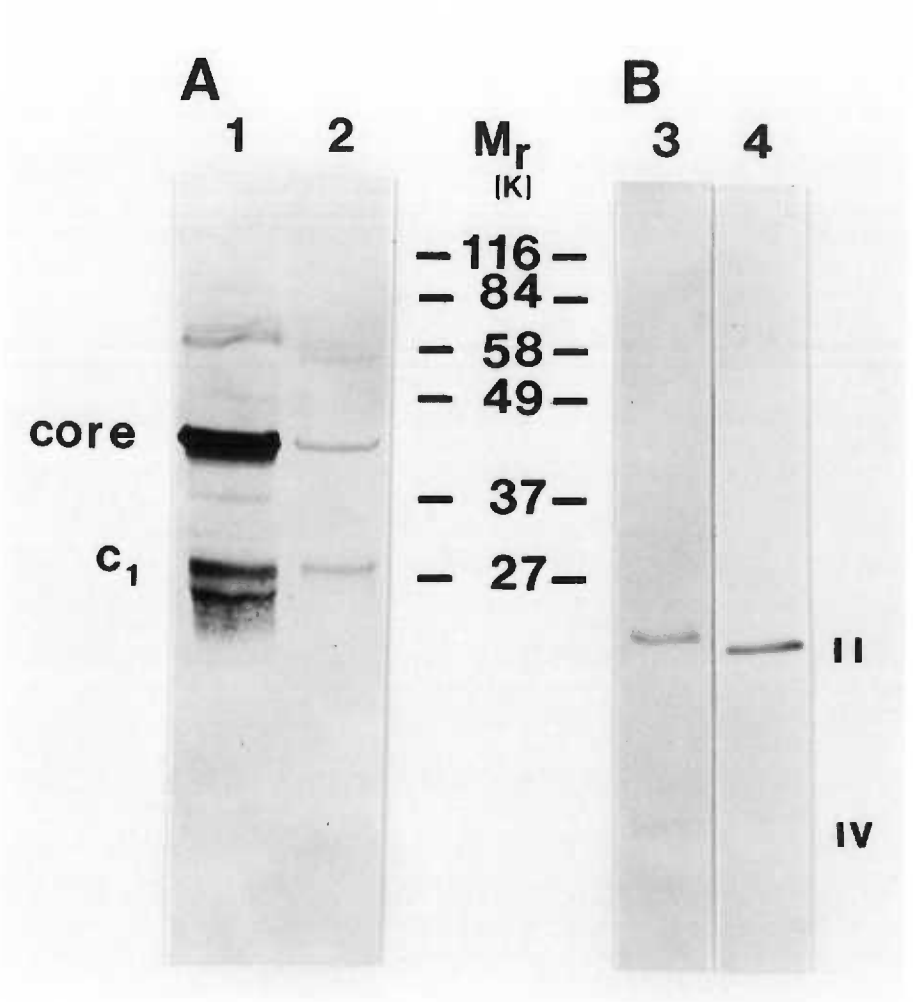
EFFECT OF OXYGEN ON THE EXPRESSION OF CYTOCHROME OXIDASE AND CYTOCHROME $\underline{b\text{-}c_1}$

CHARACTERIZATION OF THE ANTIBODIES

Cytochrome $\underline{b\text{-}c_1}$. Polyclonal antisera directed against the holoenzyme of cytochrome $\underline{b\text{-}c_1}$ specifically reacted with one of the core proteins and cytochrome $\underline{c_1}$ of AKR2B cells and beef heart mitochondria (Fig. 1, lanes 1 and 2). The identification of these subunits was based on comparisons of their apparent molecular weights (M_r) with published M_r for these proteins (Bell and Capaldi, 1976; Gellerfors and Nelson, 1981; Schägger et al, 1986; Capaldi et al, 1988).

Cytochrome Oxidase. Polyclonal antisera directed against the holoenzyme of cytochrome oxidase recognized two proteins in crude mitochondria from AKR2B cells (Fig. 1, lane 3). The antisera reacted strongly with subunit II of cytochrome oxidase and weakly with a second protein, most likely subunit IV. The identity of subunit II was based on its (1) apparent M_r, (2) comigration with subunit II of purified beef heart cytochrome oxidase, and (3) reaction with an antibody specific for cytochrome oxidase subunit II (a generous gift of Dr. R. Capaldi) on an immunoblot. The protein identified as subunit IV on the immunoblot comigrates with subunit IV of purified beef heart cytochrome oxidase on acrylamide gels of the same concentration used to make the immunoblot. Subunit II of AKR2B cells (Fig. 1, lane 1)

Fig. 1. Identification of the antigenic subunits of cytochrome $\underline{b\text{-}c_1}$ and cytochrome oxidase. Mitochondria were prepared from AKR2B cells (as described in Experimental Methods, Chapter Three), separated on 12.5% SDS polyacrylamide gels using a continuous buffer system, and immunoblotted on nitrocellulose paper. Samples of purified beef heart mitochondria were immunoblotted for comparison. The blots were probed with polyclonal antisera directed against either cytochrome $\underline{b\text{-}c_1}$ (A) or cytochrome oxidase (B). Antigenic bands were reacted with a second antibody conjugated to horseradish peroxidase and visualized by a color reaction with the conjugated substrate. Lanes 1 and 4, beef heart mitochondria; lanes 2 and 3, mitochondria from AKR2B cells. The positions of Coomassie blue stained molecular weight markers (M_r) are indicated.


A
B
1
2
3
4
M_r
[K]
116
84
58
49
37
27
core
c_1
II
IV

migrated slower than the corresponding subunit of beef heart mitochondria (Fig. 1, lane 4).

EFFECT OF OXYGEN ON THE EXPRESSION OF CYTOCHROME OXIDASE

Separation of Immunoprecipitated Cytochrome Oxidase by SDS Polyacrylamide Gel Electrophoresis

Biochemical and genetic analyses have determined that mammalian cytochrome oxidase is composed of 13 distinct proteins (Kadenbach et al, 1983; Capaldi et al, 1986; Capaldi et al, 1988; Zeviani et al, 1988). Complete separation of these proteins is dependent on the conditions of electrophoresis. Cytochrome oxidase immunoprecipitated from AKR2B cell membrane lysates was separated into seven distinct bands in a continuous buffer gel system (Maizel, 1969). These bands comigrated with those of purified beef heart cytochrome oxidase (Fig. 2). I was able to resolve 11 of the 13 subunits of cytochrome oxidase using a discontinuous gel buffer system (Schägger and von Jagow, 1987) (Fig. 3, lanes 8-9). The apparent M_r of bands separated by each gel system are consistent with the published molecular weights of the protein subunits of cytochrome oxidase (Kadenbach et al, 1983; Capaldi et al, 1986).

Subunits I, II, III, and IV were separated as distinct proteins by each method. The bands referred to as subunit V and VI in the continuous buffer gel separation (Fig. 3, lanes 3-4) were resolved further in the discontinuous buffer system (Fig. 3, lanes 8-9) into five distinct bands (referred to as subunits Va,Vb, VIa, VIb, and VIc by Kadenbach et al, 1983). Each gel system resolved a single protein

Fig. 2. Separation of immunoprecipitated cytochrome oxidase from AKR2B mouse embryo fibroblasts.

Samples of cytochrome oxidase immunoprecipitated from AKR2B cell membrane lysates labeled with [^{35}S] methionine (lane 1) or purified from beef heart (lane 2, stained with Coomassie blue) were separated by a continuous gel system. Subunits are identified by Roman numerals to the right of lane 2. The positions of [^{14}C]-labeled molecular weight markers (M_r) are indicated.

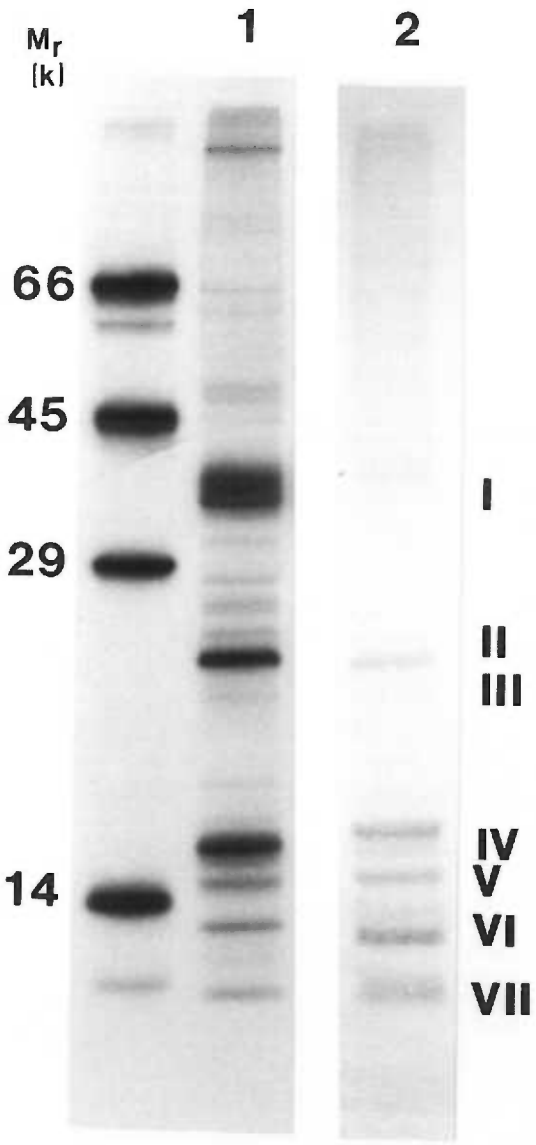

M_r [k]
1
2
66
45
29
14
I
II
III
IV
V
VI
VII

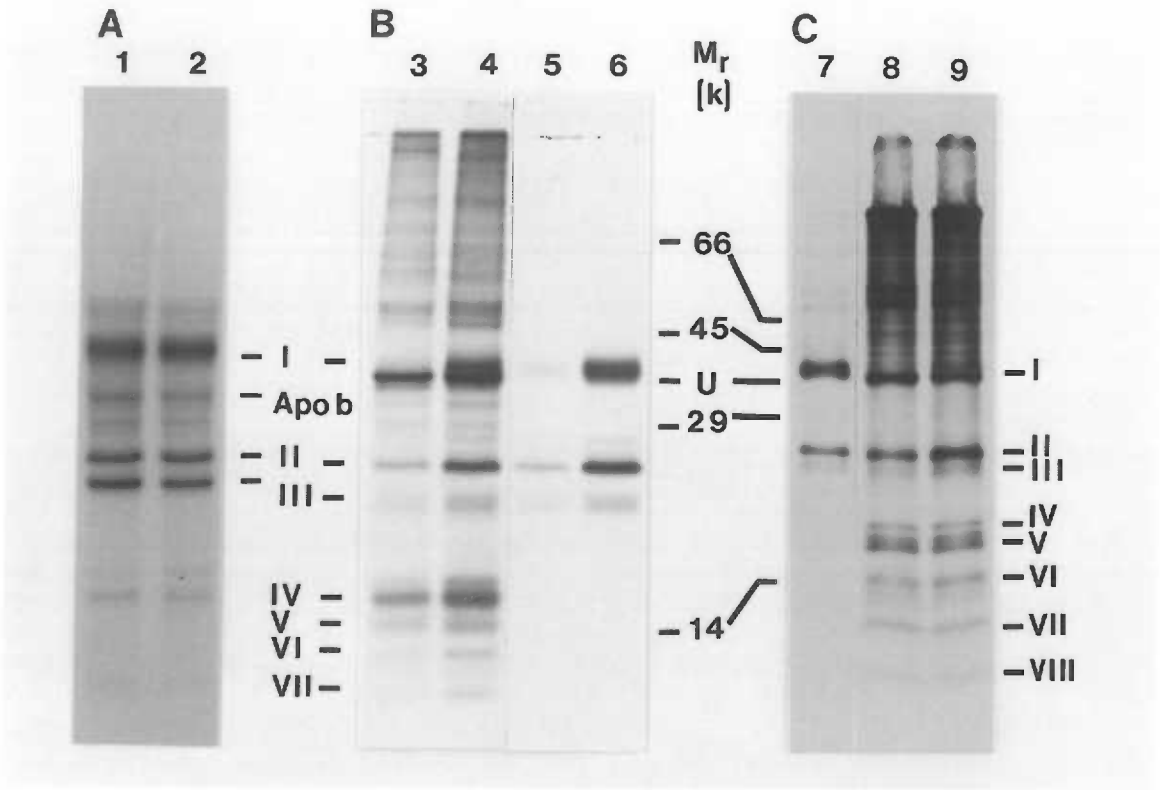
Fig. 3. Effect of oxygen on the synthesis of the mitochondrially translated subunits of cytochrome oxidase (A) and the assembly of cytochrome oxidase (B and C) in AKR2B fibroblasts.

A. Cells were grown for 24h in control (lane 1) or low (lane 2) oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 1-2h, and harvested immediately after the pulse. Cell membranes were prepared and dissociated in SDS sample buffer as described under Experimental Methods, Chapter Three, normalized to cell number in the low oxygen sample, and separated on 12.5% polyacrylamide gels with the continuous buffer gel system.

B. Cells were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of cycloheximide, and chased in methionine-supplemented, label free medium for 2.5h. The cells were labeled and chased in the presence of the appropriate test gas. Cell membrane lysates were prepared and immunoprecipitated with antisera directed against the holoenzyme of cytochrome oxidase as described under Experimental Methods, Chapter Three. Samples in lanes 3 and 4 were normalized to TCA precipitable counts in the postmembrane supernatant fraction from low oxygen cells; samples in lanes 5 and 6 were normalized to cell number in the low oxygen sample. The samples in lanes 3-6 were separated on a 12.5% SDS polyacrylamide gel using the continuous buffer system.

C. Cell membrane lysates from cells grown and prepared as described under B, above, were separated on a 16% SDS polyacrylamide gel using the discontinuous buffer system. Lane 7, cytochrome oxidase immunoprecipitated from cells labeled in the presence of cycloheximide; lane 8 (low oxygen) and lane 9 (control oxygen) cytochrome oxidase immunoprecipitated from cells labeled in the absence of cycloheximide. Samples in lanes 8 and 9 were normalized to cell number in the low oxygen sample.

The positions of mitochondrially-translated subunits (cytochrome oxidase I-III and apocytochrome *b*) and the cytoplasmically translated subunits (IV-VIII) of cytochrome oxidase and [^{14}C]-labeled molecular weight markers (M_r) are indicated. The position of an unidentified protein (U) that is coprecipitated with the cytochrome oxidase holoenzyme from cells labeled in the absence of cycloheximide (lanes 3, 4, 8, 9) is also marked.


A
1
2
B
3
4
5
6
C
7
8
9
Mr
[k]
I
Apo b
II
III
IV
V
VI
VII
66
45
U
29
14
I
II
III
IV
V
VI
VII
VIII

band with an apparent M_r consistent with that of subunit VII (Fig. 3, lanes 3-4 and 8-9). Kadenbach et al (1983) has resolved such a band further into 3 distinct proteins that he refers to as subunits VIIa, VIIb, and VIIc. A band whose apparent M_r is consistent with the known M_r of cytochrome oxidase subunit VIII was separated by the discontinuous buffer gel method (Fig. 3, lanes 8-9) but not by the continuous buffer gel method (Fig. 3, lanes 3-4)..

An unidentified band (35kDa, Fig. 3, lanes 3-4; 38kDa, Fig. 3, lanes 8-9) was coprecipitated with cytochrome oxidase holoenzyme from cell membrane lysates. The band was not found in immunoprecipitates prepared from cycloheximide-treated cells (Fig. 4, lanes 5-7), showing that the polypeptide is not translated on mitoribosomes. It was possible to separate the absorbance peaks of cytochrome oxidase subunit I from that of this unknown protein on densitometric scans of the fluorograph.

The absence of two of the identified subunits of cytochrome oxidase in these immunoprecipitates may be due to a lack of resolving power in the gels, a low abundance or absence of methionine residues in these subunits (leading to little or no uptake of radiolabeled precursor), or it may be a valid indication that the cytochrome oxidase made by AKR2B fibroblasts is composed of only 11 subunits. I did not pursue this question further. My purpose, instead, was to compare the pattern and content of proteins in immunoprecipitates from cells exposed to low O_2 to that of proteins expressed in immunoprecipitates from control O_2 cells.

Effect of O_2 on the Assembly of Cytochrome Oxidase

Cell membrane lysates prepared from cells grown for 24h in low and control O_2 and pulse labeled under the same conditions were immunoprecipitated with polyclonal antisera directed against the cytochrome oxidase holoenzyme. Samples were normalized to cell number in the low O_2 sample and separated by SDS polyacrylamide gel electrophoresis. In three experiments, reduced levels of subunits I, II, and III (the mitochondrially translated subunits) were coprecipitated with the holoenzyme from low O_2 cells (Fig. 3, lanes 3-4, 8-9; Table 1). The effect was similar whether cells were labeled in the presence or absence of cycloheximide indicating the drug did not have independent effects on the assembly of the holoenzyme.

The magnitude of the effect of O_2 on the relative specific activities of labeled subunits I, II and III in the immunoprecipitated enzyme varied from one experiment to the next (Table I). The variability was not due to experimental error in sample preparation because replicate samples prepared within the same experiment (i.e., immunoprecipitates from cells labeled in the presence or absence of cycloheximide) gave similar results.

The reduced specific activity of subunits I, II and III in the immunoprecipitated enzyme from low O_2 cells could have resulted from a defect in their synthesis or the stable assembly of the pulse-labeled proteins into the cytochrome oxidase holoenzyme. I found that exposure of cells to low O_2 tensions was associated with reduced translation of these subunits in some experiments (Table II). At least in the case of

Table I. Effect of oxygen on the assembly of cytochrome oxidase in AKR2B cells. Cells from three separate experiments were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence (+) or absence (-) of cycloheximide (CHI) in the presence of the appropriate test gas for 2h, and chased in methionine-supplemented, label free medium in the presence of low or control oxygen for 2.5h. Cell membrane lysates were prepared and immunoprecipitated with antisera directed against the holoenzyme of cytochrome oxidase as described under Experimental Methods, Chapter Three. Samples were normalized to the number of cells in the low oxygen sample and separated by SDS polyacrylamide gel electrophoresis. The absorbances of identified polypeptides on the fluorographs were determined with a densitometer as described under Experimental Methods, Chapter Three. The data are areas under specified peaks of the low oxygen sample expressed as a percent of control. The fluorograph shown in Fig. 3, lanes 5-6 corresponds to the gel analyzed in experiment 2 (+ CHI); the fluorograph in Fig. 3, lanes 8-9 corresponds to the gel analyzed in experiment 3 (- CHI). Cell-specific TCA precipitable [^{35}S] methionine counts were determined (as described under Experimental Methods, Chapter Three) in postmembrane supernates.

Subunit	Experiment Number 1	2		3	
	-CHI	+CHI	-CHI	+CHI	-CHI
Mitochondrially Translated Subunits:					
I	15%	10%	6%	39%	31%
II	15	24	27	61	54
III	-	29	37	57	49
Cytoplasmically Translated Subunits:					
IV	110		51		100
V	92		47		90
VI	50		44		108
VII	-		-		114
VIII	-		-		115
Cell-Specific Radioactive Counts in PMS*	81%		70%		98%

*PMS, postmembrane supernate

Table II. Effect of oxygen on the synthesis of the mitochondrially translated subunits of cytochrome oxidase (I, II, III) in AKR2B cells. Cells in four separate experiments were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 1-2h, and harvested immediately after the pulse. Cell membrane lysates were prepared as described under Experimental Methods, Chapter Three normalized to cell number in the low oxygen sample, and separated by SDS polyacrylamide gel electrophoresis. Fluorographs were prepared and analyzed by densitometry as described under Experimental Methods. The data are the areas under specified peaks of the low oxygen sample expressed as a percent of control. The fluorograph shown in Fig. 3, lanes 1-2 corresponds to experiment 4, below.

	Experiment Number			
Subunit	1	2	3	4
I	55%	51%	60%	100%
II	76	63	97	100
III	69	39	84	85

subunits I and II, however, the variable effect on subunit synthesis was not great enough to fully explain the reduced levels of these subunits brought down with the immunoprecipitated enzyme complex. The relative specific radioactivity (as indicated by densitometric analysis) of subunit I in immunoprecipitates prepared from low O_2 cells ranged from 6-39% of control in three experiments (Table I), whereas in four experiments, the relative incorporation of labeled amino acids into this subunit in low O_2 cells ranged from 51-100% of control (Table II); the relative appearance of subunit II in low O_2 immunoprecipitates ranged from 15-61% of control (Table I), whereas the incorporation of radiolabel into newly translated subunit II in low O_2 cells ranged from 63-100% of control (Table II). There was a comparable reduction in the synthesis and assembly of cytochrome oxidase subunit III by low O_2 cells (Tables I and II).

In one experiment, mitochondrial translation products were prepared from the same cell lysates used to prepare the immunoprecipitated enzyme complex. In this experiment, synthesis of subunits I, II, and III was 51%, 63%, and 39% of control, respectively (Table II, experiment 2). The amounts of subunits I, II, and III coprecipitated with the holoenzyme complex from these cells was 10%, 24%, and 29% of control, respectively (Table 1, experiment 2, +CHI). Thus, the decrease in O_2 availability to the cells in this experiment had two independent effects on the expression of cytochrome oxidase: (1), it affected the synthesis of all three of the mitochondrially translated subunits of cytochrome oxidase and (2), it affected the assembly of subunits I and II into the holoenzyme complex.

The effect of O_2 on the synthesis of the mitochondrially translated subunits of cytochrome oxidase was variable. In four experiments, cell-specific synthesis of subunits I, II, and III by low O_2 cells varied from 39-100% of control (Table II). As mentioned previously (Chapter Four, p. 61) total protein synthesis was also quite variable in experiments conducted with AKR2B cells. For example, the cell-specific TCA precipitable [^{35}S] methionine radioactivity (primarily a measure of the effect of O_2 on cytoplasmic protein synthesis) in cells labeled in the absence of cycloheximide varied from 70-98% of control in the experiments reported in Table I. In three other experiments (not discussed in this section), cell-specific radioactivity in low O_2 cell lysates or postmembrane supernate fractions (soluble cell proteins) was 60%, 61% and 75% of control levels. The reason for the variability is not clear. Cells were exposed to low O_2 for the same amount of time in each experiment and measurements of the O_2 tension of the low O_2 cell culture medium were less than 1 1torr in each experiment.

A way of testing for the specificity of an effect on protein synthesis is to normalize experimental and control samples to total sample radioactivity. One expects that, barring any specific or differential effects on protein synthesis, the radioactivities of individual proteins will also be equal in such samples. There are difficulties in using such an approach to decipher the nature of the sometimes present effect of O_2 on protein synthesis in my experiments. One problem is that the synthesis of mitochondrial translation products

can only be studied in cells treated with cycloheximide, an inhibitor of protein translation in the cytoplasm. Another problem is that there is no accurate way to quantify the amount of sample radioactivity associated with labeled mitochondrial translation products. This is because there are so few proteins synthesized in the mitochondrion relative to the vast number that are translated in the cytosol; even a 1% leak through the cycloheximide block has a substantial impact on the total radioactivity measured in cells radiolabeled in the presence of cycloheximide. Thus, as mitochondrial translation products can only be analyzed in cells labeled in the presence of cycloheximide, and total radioactivity can only be measured in cells labeled in the absence of cycloheximide, the assumption must be made that the general effect of O_2 on protein synthesis in drug-treated cells and non-drug-treated cells is the same, and that the basis for the effect of O_2 on the translation of total protein (most of which is carried out on cytoplasmic ribososmes) is the same as the basis for its effect on the translation of mitochondrial gene products.

With these assumptions in mind, I prepared immunoprecipitated samples of cytochrome oxidase and mitochondrial translation product samples from low and control O_2 cells grown and labeled in parallel cultures in the same experiment (experiment 2, -CHI, Table I; experiment 2, Table II). The immunoprecipitated enzyme and the translation products were each prepared from the same number of cells and then solubilized in the same total volume of SDS sample buffer. Samples applied to SDS polyacrylamide gels were either normalized to the number of cells used to prepare the samples or to total

radioactivity in cells labeled in the absence of cycloheximide.

The results of this analysis (Table III) show that normalization of the low and control O_2 mitochondrial translation product samples to radioactivity in cells labeled in the absence of cycloheximide did bring the radioactivities of subunits I and II in the low O_2 samples to within control O_2 levels. Given the assumptions already mentioned, this suggests there was little specific effect of O_2 on the synthesis of these two proteins. There remained, however a substantial effect on their assembly. Note that the relative incorporation of radiolabel into subunit I increased from 51% to 81% of control and that of subunit II increased from 63% to 96% of control when the low O_2 mitochondrial translation product samples were normalized to radioactivity instead of cell number, whereas the relative activities of subunits I and II in the low O_2 immunoprecipitate only increased from 6% to 8% of control and from 27% to 34% of control, respectively, when this sample was normalized to radioactivity instead of cell number (Table III). In this experiment O_2 appears to have affected the translation, but not the assembly of subunit III of cytochrome oxidase (Table III).

In every experiment, assembly of the mitochondrially translated subunits was more affected by low O_2 conditions than was the assembly of the cytoplasmically translated subunits (Table I). In two of three experiments, there was little or no affect on the assembly of the nuclear subunits in spite of a pronounced effect on the assembly of the mitochondrially translated subunits (Table I, experiments 1 and 3). In a third experiment, there was a considerable effect on the assembly of the cytoplasmically translated subunits (Table I, experiment 2).

Table III. Comparison of the effects of oxygen on the synthesis and assembly of the mitochondrially translated subunits of cytochrome oxidase in samples normalized to either cell number or radioactivity (counts) in the low oxygen sample. Cells from the same experiment were exposed to low or control oxygen for 24h and pulse-labeled with [^{35}S] methionine in the presence or absence of cycloheximide as described in the legends of Tables I and II. Cell membrane lysates were either used directly to determine effects on the synthesis of the mitochondrial translation products or were immunoprecipitated with antisera directed against the holoenzyme of cytochrome oxidase to determine effects on the assembly of the mitochondrially translated subunits. Samples on the gel were normalized to either the number of cells used to prepare the sample or to the number of radioactive counts in the soluble protein fraction of cells labeled in the absence of cycloheximide. Fluorographs were prepared and analyzed by densitometry as described under Experimental Methods, Chapter Three. Data are the areas under specified peaks of the low oxygen sample expressed as a percent of control. The sample shown in lanes 3-4, Fig. 3, was normalized to radioactivity, and corresponds to the data under Assembly (Counts) below.

	Synthesis (Cells labeled in the presence of CHI)		Assembly (Cells labeled in the absence of CHI)	
Normalized to:	Cells	Counts*	Cells	Counts
Mitochondrially Translated Subunits				
I	51%	81%	6%	8%
II	63	96	27	34
III	39	57	37	44

*Normalized to total radioactivity (CPM) in sample labeled in the absence of CHI

Effect of O_2 on the Metabolic Turnover of Cytochrome Oxidase

The content of pre-assembled cytochrome oxidase was not affected by growth of AKR2B fibroblasts in low O_2 for 24h (Fig. 4. A). After cell passage and attachment, cells were pulse-labeled with [^{35}S] methionine for 2h in room air. The cells were chased in methionine-supplemented label free medium in room air for 2h (to allow for normal assembly of the holoenzyme complex) and then for an additional 24h in the presence of low and control O_2 (to assess the effect of O_2 on turnover of the pre-assembled enzyme). Cell membrane lysates were immunoprecipitated with polyclonal antisera directed against the holoenzyme. Aliquots of the immunoprecipitates were normalized to the number of cells present at the beginning of the chase and were separated by SDS polyacrylamide gel electrophoresis (Maizel, 1969) and fluorographed. Densitometric analysis of the low O_2 and control O_2 lanes revealed no differences in any of the subunit bands.

The relative stability of enzyme made and assembled in low O_2 was not determined because synthesis of new enzyme was impaired in cells grown under low O_2 (see above).

Effect of O_2 on the Degradation and Stability of the Mitochondrial Translation Products

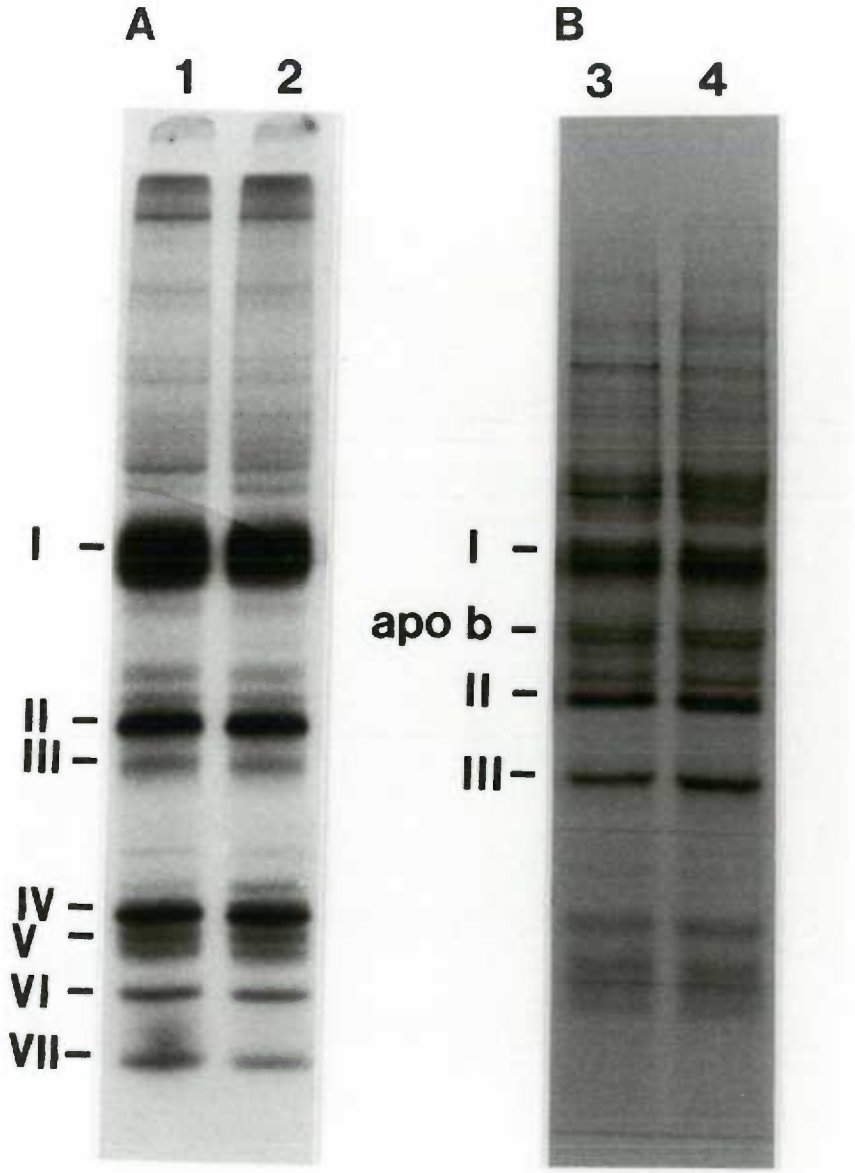
Since more of the mitochondrially translated subunits of cytochrome oxidase were made than were coprecipitated with the holoenzyme from low O_2 cells (subunits I and II, in particular), the fate of unassembled protein subunits is unknown. These subunits in low O_2 cells may be degraded or may remain in the cells in an unassembled form.

Fig. 4. Effect of oxygen on the turnover of cytochrome oxidase and mitochondrial translation products in AKR2B mouse embryo fibroblasts.

A. Turnover of cytochrome oxidase. Cells were labeled in room air; chased for 2h in room air to allow for normal assembly of cytochrome oxidase; and then chased for an additional 24h in either low or control oxygen. Roman numerals to the left of lane 1 indicate the subunits of cytochrome oxidase.

B. Mitochondrial translation products. Cells were labeled in low or control oxygen and then chased for 24h in low or control O_2. I, II, and III are the mitochondrially translated subunits of cytochrome oxidase; apo b is the apocytochrome b subunit of cytochrome $b\text{-}c_1$.

See text for methodology. Lanes 1 and 3, low oxygen samples; lanes 2 and 4, control oxygen samples.


A
1
2
I
II
III
IV
V
VI
VII
B
3
4
I
apo b
II
III

To see if mitochondrially translated proteins from low O_2 cells were degraded, cells were grown in either low or control O_2 for 15h and then pulse labeled with [^{35}S] methionine for 2h in the presence of cycloheximide and the appropriate test gas. Some cells were harvested immediately after the pulse to examine the effects of O_2 on the synthesis of the mitochondrial translation products and some cells were harvested after a 24h chase in label free medium in low or control O_2 to examine the effects of O_2 on degradation of the labeled proteins. I observed only a slight reduction in the specific radioactivities of the low O_2 proteins (relative to the specific radioactivities present in similar subunits of the control O_2 sample) (Fig. 4, B; Table IV), an indication that subunits I and II were not degraded faster in low O_2 cells than they were in control O_2 cells. The reduction in the specific radioactivity of subunit I from low O_2 cells (Table IV) can be accounted for by the decrease in its synthesis in these cells (the specific radioactivity of subunit I was 80% of control in this experiment, data not shown).

Since mitochondrially translated proteins from low O_2 cells remained in whole membrane lysates 24h after labeling, but were reduced in the immunoprecipitated enzyme, one might expect to see increased levels of these unassembled subunits in fractionated samples prepared from low O_2 cells. To address this question, labeled proteins in low and control O_2 (1), postmembrane supernates (soluble protein cell fraction), (2), post-lysis pellets (Triton X-100 insoluble material in the cell membrane fraction), and (3), the post immunoprecipitate supernates (cell membrane proteins not recognized by the polyclonal

Table IV. Degradation of mitochondrial translation products (cytochrome oxidase subunits I, II, III and apocytochrome *b*) in low and control oxygen cells. Cells were grown for 15h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 2h; and chased for 24h in methionine-supplemented label free medium in the presence of low and control oxygen. Cell membrane lysates were prepared as described under Experimental Methods and separated on an SDS polyacrylamide gel. Samples applied to the gel wells were normalized to cell number in the low oxygen sample at the beginning of the chase. Radioactive counts in identified bands excised from the dried gel were determined as described under Experimental Methods, Chapter Three. Data are number of counts in the low oxygen gel slices expressed as a percent of those present in controls.

Subunit	Percent of Control
I	84%
II	97
III	93
Apo *b*	98

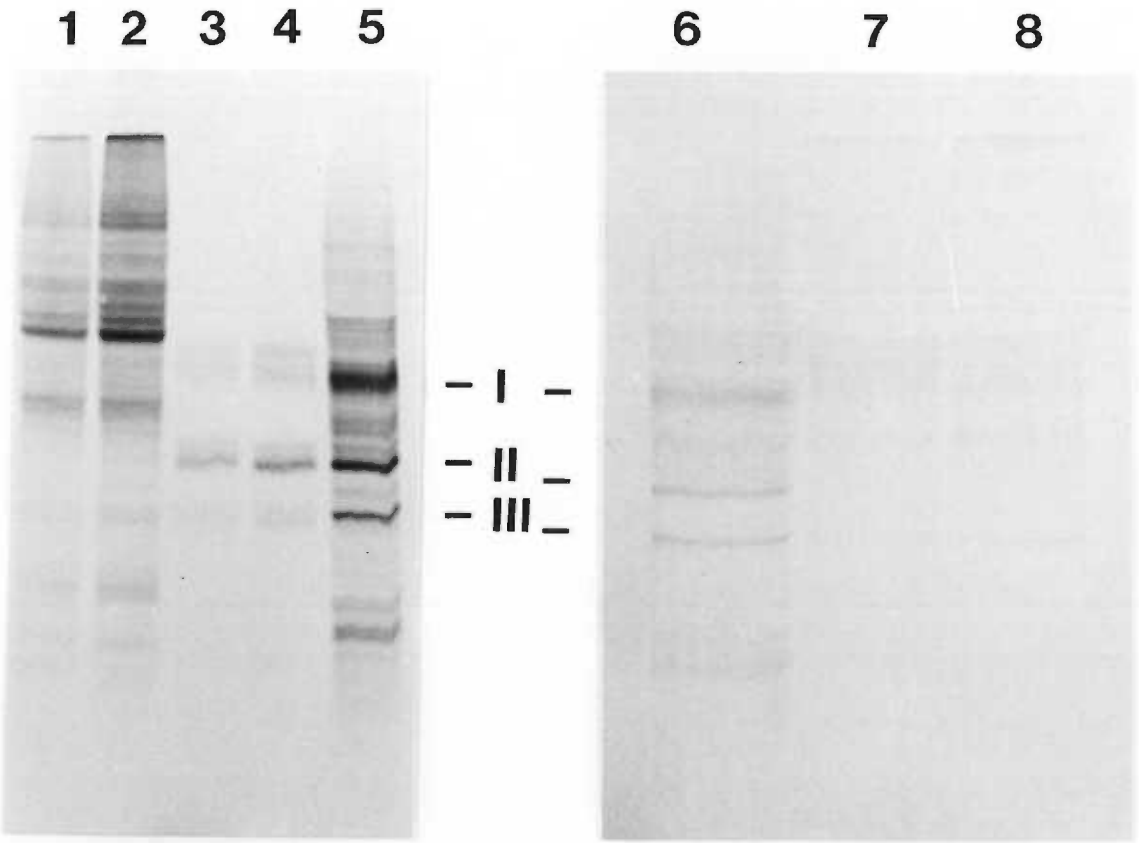
antibody directed against cytochrome oxidase) were separated on SDS polyacrylamide gels. All samples were normalized to cell number in the low O_2 sample. 25% of each sample was applied to the gel lanes in Fig. 5. Subunits I and II were not detected in the postmembrane supernates (Fig. 5, lanes 1 and 2) indicating these subunits were not lost during preparation of the cell membrane pellet. A protein band that comigrated with authentic cytochrome oxidase subunit I was present in both the post-lysate pellets (data not shown) and the post immunoprecipitate supernates (Fig. 5, lanes 7 and 8) of low and control O_2 cells; there was, however, no enrichment for this protein in either fraction from the low O_2 cells. Subunit II of cytochrome oxidase was absent in all cell fractions.

EFFECT OF OXYGEN ON THE EXPRESSION OF CYTOCHROME b-c_1

Separation of Immunoprecipitated cytochrome b-c_1 by SDS Polyacrylamide Gel Electrophoresis

Polyclonal antisera directed against a core protein and cytochrome c_1 of the cytochrome b-c_1 complex immunoprecipitated one major band from cells labeled with [^{35}S] methionine in the presence of cycloheximide (Fig. 2, lanes 2-3). This band comigrated with the mitochondrial translation product, apocytochrome b (Fig. 6, lane 1). Small amounts of subunits I, II, and III of cytochrome oxidase were brought down as contaminants in the immunoprecipitates from cycloheximide treated cells.

Fig. 5. Electrophoretic separation of immunoprecipitated cytochrome oxidase and proteins present in the postmembrane and post-immunoprecipitate supernatant fractions prepared from low and control oxygen cells labeled with [^{35}S] methionine in the presence of cycloheximide. Cells were grown for 24h in low or control oxygen, labeled with [^{35}S] methionine in the presence of cycloheximide for 2h, and chased for 2.5h in the presence of the appropriate gas. Cell membrane lysates immunoprecipitated with antisera directed against cytochrome oxidase (lanes 3-4), and the post-membrane supernates (lanes 1-2) and post-immunoprecipitate supernates (lanes 7-8) were prepared as described under Experimental Methods, chapter 3. The cell fractions were prepared from cells exposed to either low or control O_2 in the same experiment, and the samples were all normalized to the number of cells in the low oxygen cultures. Samples were separated on 12.5% SDS polyacrylamide gels. Samples of mitochondrial translation products were separated (lanes 5-6) for subunit identification. The positions of subunits I, II, and III of cytochrome oxidase are indicated. Lanes 1, 3, and 7, low oxygen samples; lanes 2, 4, and 8, control oxygen samples.


1 2 3 4 5
6 7 8
I
II
III

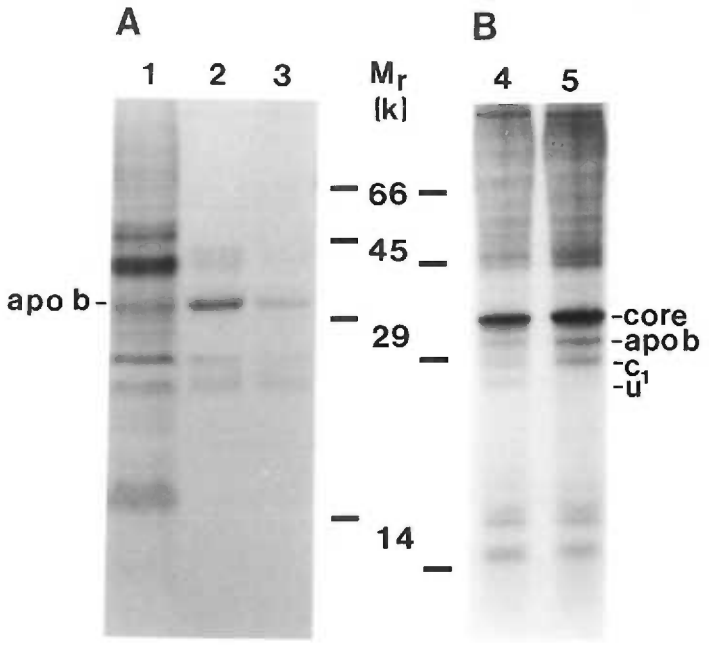
I identified three subunits of cytochrome $\underline{b}$-$\underline{c}_1$ in immunoprecipitates from cells labeled in the absence of inhibitor (Fig. 6, lanes 4-5). As observed by others, core protein II runs just ahead of the immunoglobulin heavy chain protein (Bell et al, 1979). The relative mobilities of the subunits identified as cytochrome $\underline{b}$ and cytochrome $\underline{c}_1$ are consistent with those reported for the purified beef heart enzyme (Bell and Capaldi, 1976; Schägger et al, 1986; Capaldi et al, 1988). The three low molecular weight polypeptides on the gel (Fig. 6, lanes 4-5) have higher relative mobilities than any of the six described low molecular weight polypeptides of purified beef heart cytochrome $\underline{b}$-$\underline{c}_1$ (Bell and Capaldi, 1976; Schägger et al, 1986; Capaldi et al, 1988), although, the lowest band comigrates with a band of M_r 17,000 present in a separation of purified beef heart cytochrome $\underline{b}$-$\underline{c}_1$ on my gels (data not shown).

The identity of the band below subunit $\underline{c}_1$ in the low O_2 sample is not known (Fig. 6, lane 4). Its absence from the control O_2 immunoprecipitate suggests it is a precursor or breakdown product of one of the other subunits of the complex that specifically forms or accummulates in cells grown in low O_2. Interestingly, a band of similar molecular weight has been observed in electrophoretic separations of the $\underline{b}$-$\underline{c}_1$ complex from heme-deficient mutant yeast; this polypeptide is absent from complexes assembled by heme-sufficient mutants or wild type yeast (Lin et al, 1982).

Fig. 6. Comparison of the assembly of cytochrome $\underline{b}$-c_1 in low and control oxygen cells. In two separate experiments (A and B), cells were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence (lanes 1-3) or absence of cycloheximide (lanes 4-5) in the presence of the appropropriate test gas for 2h and either harvested immediately (for analysis of the mitochondrial translation products, lane 1) or chased in methionine-supplemented, label free medium in the presence of low (lanes 3 and 4) or control (lanes 2 and 5) oxygen for 2h (for immunoprecipitation of the assembled enzyme complex). Cell membrane lysates were prepared and immunoprecipitated with antisera directed against the holoenzyme of cytochrome $\underline{b}$-c_1 as described under Experimental Methods, Chapter Three. Samples in lanes 2-5 were normalized to the number of cells in the low oxygen sample. All samples were separated on 12.5% polyacrylamide gels using the continuous buffer gel system (24). The positions of [^{14}C]-labeled molecular weight markers (M_r) are indicated.

A. The cytochrome $\underline{b}$ subunit of the immunoprecipitated enzyme (lanes 2-3) comigrates with apocytochrome $\underline{b}$ in the mitochondrial translation product separation (lane 1).

B. The positions of core protein II, cytochromes $\underline{b}$ and $\underline{c}_1$ are indicated. The location of an unidentified protein (U), present only in the low oxygen sample (lane 4), is also shown.


A
B
1
2
3
4
5
M_r (k)
66
45
29
14
apo b
core
apo b
c_1
u^1

Effect of O_2 on the Assembly of Cytochrome b-c_1

In two experiments (in which enzyme was immunoprecipitated from cells labeled in the presence of cycloheximide), the amount of apocytochrome b immunoprecipitated by anti-cytochrome b-c_1 antiserum from low O_2 cells was 30% (or less) of that from control cells (Fig. 6, lanes 2-3, and one other unreported experiment). Similarly, in a third experiment, (in which enzyme was immunoprecipitated from cells labeled in the absence of cycloheximide), the apocytochrome b subunit in low O_2 cells was 29% of control cells (Fig. 6, lanes 4-5).

In contrast, there was only a slight reduction in the translation of apocytochrome b by low O_2 cells (Table V), suggesting that low O_2 selectively affects the assembly of apocytochrome b into the cytochrome b-c_1 complex in these cells.

As shown previously, relative to control cells, there was little difference in the amount of degradation of newly-synthesized apocytochrome b over a 24h period (Table IV).

O_2 availability affected the assembly of some, but not all, nuclear encoded subunits of cytochrome b-c_1 (Fig. 6, lanes 4-5). The core II protein subunit was 70% of control and the cytochrome c_1 subunit was 32% of control in the low O_2 immunoprecipitate. There were no quantitative differences in the amounts of the three low molecular subunits immunoprecipitated from low and control O_2 membrane lysates. In this experiment, protein synthesis in low O_2 cells was 61% of that in control cells (as indicated by a comparison of the TCA precipitable [^{35}S] methionine counts in the postmembrane supernates).

Table V. Effect of oxygen on the synthesis of apocytochrome *b*. Cells in four separate experiments were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 1-2h, and harvested immediately after the pusle. Cell membrane lysates were prepared as described in Chapter Three, Experimental Methods, normalized to cell number in the low oxygen sample,1 and separated by SDS polyacrylamide gel electrophoresis. Fluorographs were prepared and analyzed by densitometry as described in Chapter Three. The data are the areas under specified peaks of the low oxygen sample expressed as a percent of control. The fluorograph shown in Fig. 3, lanes 1-2, corresponds to experiment 4, below.

Experiment Number			
1	2	3	4
87%	66%	82%	72%

CHAPTER SIX

DISCUSSION

In this study, AKR2B fibroblasts grown at a relatively low O_2 tension (X ± SD medium O_2 tension = 7 ± 4 torr) for 24h doubled once and exhibited about half of the cytochrome oxidase activity present in cells grown under control O_2 conditions (X ± SD medium O_2 tension = 137 ± 24 torr). As might be expected, the low O_2 cells also exhibited a lower rate of cellular O_2 consumption than the control O_2 cells. Several observations suggested the comparative decrease in enzyme activity in low O_2 cells was due to a decrease in the synthesis or assembly of a functional enzyme complex and a dilution of pre-existing enzyme by increased cell number. First, aeration of the low O_2 cells restored control levels of enzyme activity, but only after 2-24h; and second, there was no evidence for increased turnover of pre-assembled holoenzyme in the low O_2 cells.

Immunoprecipitation studies showed a relative deficiency of radiolabeled protein in newly assembled cytochrome oxidase from low O_2 cells. The most striking result was a consistent, relative, reduction in the specific radioactivities of subunits I, II, and III (mitochondrially translated proteins) in enzyme immunoprecipitates prepared from low O_2 cells. Although the relative incorporation of labeled amino acids into these proteins was reduced in some experiments, decreased availability of newly-synthesized subunits does

not appear to have been sufficient to explain the reduced specific activities of subunits I and II in the holoenzyme complex immunoprecipitated from low O_2 cells. This conclusion is strengthened by the results of an experiment in which both translation and assembly were studied in the same cells. Although synthesis of subunits I and II in low O_2 cells was reduced to about half of that in control O_2 cells, assembly of these pulse-labeled subunits in the low O_2 holoenzyme was reduced even further to only 24% or less of that in the control O_2 sample. These results suggest that O_2 exerted an effect on the assembly of subunits I and II into the cytochrome oxidase complex that was independent of an effect on their synthesis. The basis for the reduced appearance of labeled subunit III in the holoenzyme complex is not clear.

Unassembled cytochrome oxidase subunits I and II that were newly synthesized by low O_2 cells were not degraded or lost during preparation of the cell membrane pellet; they were just as susceptible to mild detergent lysis as control O_2 subunits; and they were detectable by anti-cytochrome oxidase antisera. A reasonable explanation for their absence in the low O_2 immunoprecipitated enzyme complex and in the low O_2 cell fractions is that these proteins were unstably assembled in cells grown under low O_2 tensions; and, that as a result of their loose association with other subunits or prosthetic groups in the holoenzyme complex, they were dislodged and lost during preparation of the immunoprecipitated enzyme (i.e., during the washing steps). I did not test this possibility because proteins ending up in the immunoprecipitate washes would be too dilute to analyze effectively.

Compared with its effect on the mitochondrially translated subunits, the effect of O_2 on the nuclear encoded subunits of cytochrome oxidase was minimal. In two experiments, there was little or no reduction in the relative specific activities of nuclear gene products coprecipitated with antisera directed against the holoenzyme complex; in a third experiment, there was a considerable reduction in these pulse-labeled subunits in the immunoprecipitated enzyme. Notably, there was a substantial reduction in total protein synthesis in the low O_2 cells in the latter experiment. One interpretation of these data, then, is that decreased O_2 availability did not specifically inhibit the synthesis, import or assembly of the nuclear encoded subunits of cytochrome oxidase. Instead, when a depletion of labeled subunits was observed in the immunoprecipitated enzyme, it may have been a consequence of a general (and variable) effect of O_2 on overall protein synthesis in AKR2B cells.

O_2 availability affected the expression of cytochrome $\underline{b}$-$\underline{c}_1$ in a manner that was similar to its effect on cytochrome oxidase. Assembly of labeled apocytochrome $\underline{b}$ (a mitochondrial translation produce) was reduced in the low O_2 immunoprecipitated complex relative to controls, and the reduction was not explained by an effect of O_2 on the synthesis of this protein. There was little effect of O_2 on assembly of core protein or one other unidentified nuclear encoded subunit, but there was a substantial effect on the assembly of the cytoplasmically translated cytochrome $\underline{c}_1$ protein. I do not know whether synthesis of this subunit was affected. It is possible, however, that since

assembly of both apocytochrome *b* and cytochrome c_1 was affected, the defective assembly of this complex could have been linked with an effect of O_2 on the synthesis or incorporation of the heme *b* and heme c_1 prosthetic groups into the *b*-c_1 complex.

O_2 availability had a variable effect on the synthesis of both nuclear and mitochondrial encoded proteins in this study. Since measurements of the O_2 tension of the cell culture medium are general and do not describe the true O_2 tension at the cell surface, it is possible that local differences in O_2 tension at the level of the cell monolayer were an important source of this variability. In addition, if the critical O_2 tension for protein synthesis in these cells lies within the range of low O_2 tensions produced in my experiments, then even a slight increase or decrease in O_2 availability to the low O_2 cells could result in substantial changes in the amount of labeled products. Another possibility is that the metabolism of cells from different freezer stocks may have differed or that the metabolism of cells changed as the cultures aged (i.e., as the passage number increased). I tried to guard against this by thawing out fresh cultures every one or two months. I was also careful not to let the cultures become overly confluent before they were passed, but it is still possible that selection for certain metabolic phenotypes may have occurred in small, local areas of high cell density.

As alluded to previously, the inconsistent results obtained from the immunoprecipitation studies may have been a consequence of the potential, coincidental, effect of O_2 on the translation of both mitochondrial and nuclear gene products. This is evident in that

effects of O_2 on assembly (in particular, of subunits I and II and the nuclear subunits of cytochrome oxidase) were greater in experiments where effects on total protein synthesis were observed (Chapter Five, Table I, p. 95). In addition, when the effect of O_2 on the synthesis of subunits I and II is subtracted out, there remains a substantial effect on the assembly of these two proteins (see above). Together, these observations suggest that when present, a general effect of O_2 on overall protein synthesis could be masking a more specific effect of O_2 on assembly of this enzyme complex. Hence, variability in the assembly data could simply be a function of the variable effect of O_2 on protein synthesis in these cells. Alternatively, or additionally, a variable dissociation of subunits from an unstably assembled enzyme complex in low O_2 cells could explain the inconsistent result obtained from the immunoprecipitation studies.

It is interesting that in some experiments, O_2 availability affected the quantitative output of two physically-separated translation systems. Perhaps this is not surprising if one considers that the activities of these two genetic systems must, by necessity, be coordinated. All of the RNA components required for the synthesis of mitochondrially-derived proteins are encoded by mitochondrial DNA, but the protein factors necessary for mRNA translation and post-transcriptional processing are products of the nucleo-cytoplasmic synthesizing apparatus (Attardi and Schatz, 1988). Thus, a general effect of O_2 on cytoplasmic protein synthesis could be expected to have repercussions on mitochondrially-directed protein synthesis. Support

for such an interaction between the nucleus and the mitochondria has been provided by studies of yeast nuclear gene mutants (pet mutants; McEwen et al, 1986) and studies demonstrating that the accumulation of several of the mitochondrially encoded proteins is dependent on the presence of specific nuclear gene products (reviewed in Fox, 1986). Mutants whose phenotypes have been examined indicate that (1) at least three nuclear gene products, PET122, PET54, and PET494, are specifically involved in activating the translation of subunit III of cytochrome oxidase (Costanzo and Fox, 1988); (2) at least three nuclear encoded proteins, PET111, PET112, and SCO1, are necessary for the post-transcriptional processing of subunit II of cytochrome oxidase (Poutre and Fox, 1987; Schulze and Rödel, 1988); and (3) defective expression of the nuclear MSS511 gene is associated with defective processing of the cytochrome oxidase subunit I mRNA transcript (Simon and Faye, 1984). A preliminary observation that is pertinent to this study, is that expression of PET494 is repressed more than 5-fold in anaerobically grown yeast (Fox, et al, 1988).

The similarity of the effect of O_2 on the assembly of cytochrome oxidase and cytochrome $\underline{b}$-$\underline{c}_1$ in AKR2B cells suggests a common mechanism. One possibility is that a deficiency of heme $\underline{b}$, heme $\underline{a}$, or heme $\underline{c}_1$ in O_2-deprived cells mediates the effect. Two lines of evidence support this notion. First, O_2 is a substrate in two described reactions of the heme $\underline{b}$ biosynthetic pathway (Poulson and Polglase, 1974; Poulson and Polglase, 1975; Poulson, 1976), and although the reactions that lead to the production of heme $\underline{a}$ and heme $\underline{c}_1$ have not been characterized, it is possible that other O_2-utilizing enzymes are also

required for the synthesis of these metaloproteins (H. Mason, personal communication). Second, heme *b* appears to be required for the assembly of fetal rat liver cytochrome oxidase (Woods, 1977) and heme *a*, but not heme *b*, induces the assembly of the three mitochondrially translated subunits of cytochrome oxidase in isolated rat liver mitochondria (Wielburski and Nelson, 1984).

O_2 and heme availability have also been observed to affect the assembly of cytochrome oxidase and cytochrome *b-c*$_1$ in yeast. O_2 is required for the assembly of subunits I and II with subunits VI and VII of yeast cytochrome oxidase (Woodrow and Schatz, 1979), and heme has been shown to control the assembly of yeast cytochrome oxidase (Saltzgaber-Müller and Schatz, 1978) and cytochrome *b-c*$_1$ (Lin et al, 1982).

It is known that yeast are heme deficient when grown under strict anaerobiasis (Lukaszkiewicz and Bilinski, 1979). I do not know whether heme availability was limited in AKR2B cells grown under the relatively low O_2 tensions produced in this study, but several published observations suggest that it may have been. (1) The *in vitro* Km_{O_2} of protoporphyrinogen oxidase (isolated from mouse liver) in the presence of saturating amounts of protoporphyrin is 125μM (Ferreira and Dailey, 1988). The Km_{O_2} of this enzyme *in vivo* (where the amount of protoporphyrin is never very high) is not known; however, the value determined *in vitro* suggests that O_2 could be rate limiting for this enzyme under physiological conditions. (2) Heme synthesis in avian red blood cells is depressed when the O_2 content of whole blood is less

than 7% and is not observed when available O_2 is less than 1% (Falk et al, 1959).

Supplementation of AKR2B cells with 10μM hemin (heme *b*) did not restore normal levels of cytochrome oxidase activity to cells grown in low O_2 (data not shown). This result is ambiguous, however, as I do not know whether hemin was taken up by the mitochondria and if so, whether it was converted to heme *a*.

In addition to its effects on the expression of cytochrome oxidase and cytochrome *b*-c_1, O_2 availability had other effects on AKR2B cells. I have already discussed the variable effect of O_2 on overall protein synthesis in these cells. In addition O_2 availability affected cell proliferation. Cells grown under relatively low O_2 tensions for 24h continued to divide, but reached lower densities than the control O_2 cells. Between 24h and 48h, there was no net change in cell number in the low O_2 cultures. In contrast, the control O_2 cells proliferated logarithmically throughout 48h of growth (Table I, Chapter Four). A similar inhibition of cell proliferation at relatively low O_2 tensions has been observed in other mammalian cells (including fibroblasts, epithelial cells, and smooth muscle cells) (Balin et al, 1976; Taylor and Camalier, 1982; Pentland and Marcelo, 1983; Benitz et al, 1986). One proposal to explain the effect of O_2 availability on cell division is that there is a deficiency of pyrimidine precursors in O_2-deprived cells that results from the O_2-dependence of two enzymes in the pyrimidine (deoxy)nucleotide pathway (Loffler, 1989).

The experiments in this study were designed with an awareness of the potential effects of decreased O_2 availability on cell growth and

metabolism. I tested the effects of O_2 on the synthesis and assembly of two respiratory cytochromes at a time when I was confident the cells were viable (as judged by their morphology, survival, and ability to proliferate and synthesize protein) and when cytochrome oxidase activity was sufficiently reduced to allow characterization of the response. Unfortunately, I did not anticipate the variable effect of O_2 on protein synthesis that was observed in these experiments. A better understanding of the cause of this variability is likely crucial to a complete understanding of the results from the immunoprecipitation studies and will be necessary before adequate experimental controls can be designed to separate out potential, overlapping, effects of O_2 availability on protein expression in these cells.

CHAPTER SEVEN

CONCLUSIONS

(1). AKR2B mouse embryo fibroblasts grown at low O_2 tensions ($\bar{X} \pm$ SD medium O_2 tension = 7 $\pm$ 4 torr) for 24h exhibited significantly less cytochrome oxidase activity than control cells grown at a high O_2 tension ($\bar{X} \pm$ SD mean O_2 tension = 137 $\pm$ 24 torr). Enzyme activity in the low O_2 cells returned to control levels within 24h (but not after 2h) of exposure of the low O_2 cultures to control O_2 conditions.

(2). Immunoprecipitation studies indicated a relative deficiency of radiolabeled protein in newly assembled cytochrome oxidase and cytochrome $\underline{b}$-c_1 from low O_2 cells.

(3). The results of this study suggest that O_2 availability affected the assembly of subunits I and II of cytochrome oxidase and the apocytochrome $\underline{b}$ protein of the $\underline{b}$-c_1 complex. The effect of O_2 on the assembly of these mitochondrial translation products appeared to be independent of an effect on their synthesis.

(4). Data from two experiments suggest that O_2 availability did not specifically affect the synthesis, import, or assembly of the nuclear encoded subunits of cytochrome oxidase. The decreased appearance of pulse-labeled cytoplasmically translated subunits into the low O_2 holoenzyme complex in a third experiment may reflect a general effect

of reduced O_2 availability on protein synthesis in AKR2B cells irrespective of any specific effect on cytochrome oxidase.

REFERENCES

Acosta, D. and L. Cheng-Pei. 1979. Injury to primary cultures of rat heart endothelial cells by hypoxia and glucose deprivation. In Vitro 15:929-934.

Adebonojo, F.O., K.G. Bensch, and D.W. King. 1961. The effect of nitrogen on the enzymatic pattern of strain L cells. Cancer Res. 21:252-256.

Anderson, G.R., D.L. Stoler, and L.A. Scarcello. 1989. Normal fibroblasts responding to anoxia exhibit features of the malignant phenotype. J. Biol. Chem. 264:14885-14892.

Anderson, S., A.T. Bankier, B.G. Barrell, M.H.L. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, and I.G. Young. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457-65.

Arcangioli, B. and B. Lescure. 1985. Identification of proteins involved in the regulation of yeast iso-1-cytochrome c expression by oxygen. EMBO J. 4:2627-2633.

Asson-Batres, M.A, M.K. Stock, J.F. Hare, and J. Metcalfe. 1989. O_2 effect on composition of chick embryonic heart and brain. Respir. Physiol. 77:101-110.

Attardi, B., B. Cravioto, and G. Attardi. 1969. Membrane-bound ribosomes in HeLa cells. J. Mol. Biol. 44:47-70.

Attardi, G. and G. Schatz. 1988. Biogenesis of mitochondria. Ann. Rev. Cell. Biol. 4:289-333.

Aw, T.Y. and D.P. Jones. 1982. Secondary bioenergetic hypoxia. J. Biol. Chem. 257:8997-9004.

Aw, T.Y., E. Wilson, T.M. Hagen, and D.P. Jones. 1987. Determinants of mitochondrial O_2 dependence in kidney. Am. J. Physiol. 253:F440-F447.

Balin, A.K., D.B.P. Goodman, H. Rasmussen, and V.J. Cristofalo. 1976. The effect of oxygen tension on the growth and metabolism of WI-38 cells. J. Cell. Physiol. 89:235-250.

Bauer, C.E., D.A. Young, and B.L. Marrs. 1988. Analysis of the Rhodobacter capsulatus puf operon. J. Biol. Chem. 263:4820-4827.

Baumann, H. and D. Doyle. 1979. Localization of membrane glycoproteins by in Situ neuraminidase treatment of rat hepatoma tissue culture cells and two-dimensional gel electrophoretic analysis of the modified proteins. J. Biol. Chem. 254:2542-2550.

Bell, R.L. and R.A. Capaldi. 1976. The polypeptide composition of ubiquinone-cytochrome c reductase (complex III) from beef heart mitochondria. Biochemistry 15:996-1001.

Bell, R.L., J. Sweetland, B. Ludwig, and R.A. Capaldi. 1979. Labeling of complex III with [^{35}S] diazobenzenesulfonate: orientation of this electron transfer segment in the mitochondrial inner membrane. Proc. Natl. Acad. Sci. 76:741-745.

Benitz, W.E., J.D. Coulson, D.S. Lessler, and M. Bernfield. 1986. Hypoxia inhibits proliferation of fetal pulmonary arterial smooth muscle cells in vitro. Pediatr. Res. 20:966-972.

Block, E.R., J.M. Patel, and D. Edwards. 1989. Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes. Am. J. Physiol. 257:C223-C231.

Bradley, T.R., G.S. Hodgson, and M. Rosendaal. 1978. The effect of oxygen tension on haemopoietic and fibroblast cell proliferation in vitro. J. Cell Physiol. 97:517-522.

Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.

Capaldi, R.A., D. González-Halphen, and S. Takamiya. 1986. Sequence homologies and structural similarities between the polypeptides of yeast and beef heart cytochrome c oxidase. FEBS Lett. 207:11-17.

Capaldi, R.A., D. González-Halphen, Y-Z. Zhang, and W. Yanamura. 1988. Complexity and tissue specificity of the mitochondrial respiratory chain. J. Bioenerg. and Biomembr. 20:291-311.

Chin, C.H. 1950. Effect of aeration on the cytochrome systems of the resting cells of brewers' yeast. Nature 165:926-927.

Chodosh, L.A., J. Olesen, S. Hahn, A.S. Baldwin, L. Guarente and P.A. Sharp. 1988. A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable. Cell 53:25-35.

Clark, W.G., E. Davidson, and B.L. Marrs. 1984. Variation of levels of mRNA coding for antenna and reaction center polypeptides in Rhodopseudomonas capsulata in response to changes in oxygen concentration. J. Bacteriol. 157:945-948.

Clejan, L., D.S. Beattie, E.G. Gollub, K-P. Liu, and D.B. Sprinson. 1980. Synthesis of the apoprotein of cytochrome b in heme-deficient yeast cells. J. Biol. Chem. 255:1312-1316.

Cloud, P. 1983. The biosphere. Sci. Am. 249:176-189.

Costanzo, M.C. and T.D. Fox. 1988. Specific translational activation by nuclear gene products occurs in the 5' untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. 85:2677-2681.

Cumsky, M.G., C. Ko, C.E. Trueblood, and R.O. Poyton. 1985. Two nonidentical forms of subunit V are functional in yeast cytochrome c oxidase. Proc. Natl. Acad. Sci. 82:2235-2239.

Criddle, R.S. and G. Schatz. 1969. Promitochondria of anaerobically

grown yeast. I. Isolation and biochemical properties. Biochemistry 8:322-334.

Cunningham, F.G., P.C. MacDonald, and N.F. Gant. 1989. Williams Obstetrics. Norwalk: Appleton & Lange, p. 238.

Dannenberg, A.M., M.S. Burstone, P.C. Walter, and J.W. Kinsley. 1963. A histochemical study of phagocytic and enzymatic function of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages. I. Survey and quantitation of enzymes, and states of cellular activation. J. Cell. Biol. 17:465-486.

David, M., M-L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertig, P. Boistard, and D. Kahn. 1988. Cascade regulation of nif gene expression in Rhizobium meliloti. Cell 54:671-683.

Dawkins, M.J.R. 1959. Respiratory enzymes in the liver of the newborn rat. Proc. Royal Soc. Lond. 150:284-298.

Dennis, E.S., W.L. Gerlach, J.C. Walker, M. Lavin, and W.J. Peacock. 1988. Anaerobically reglated aldolase gene of maize. J. Mol. Biol. 202:759-767.

Dickman, K.G. and L.J. Mandel. 1989. Glycolytic and oxidative metabolism in primary renal proximal tubule cultures. Am. J. Physiol. 257:C333-C340.

Ditta, G., E. Virts, A. Palomares, and C-H Kim. 1987. The nifA gene of Rhizobium meliloti is oxygen regulated. J. Bacteriol.

169:3217-3223.

Dixon, R.A. 1984. The genetic complexity of nitrogen fixation. J. Gen. Microbiol. 130:2745-2755.

Dixon, R.A., N.C. Henderson, and S. Austin. 1988. DNA supercoiling and aerobic regulation of transcription from the Klebsiella pneumoniae nifLA promoter. Nucl. Acids Res. 16:9933-9946.

Dumont, M.E., J.F. Ernst, and F. Sherman. 1988. Coupling of heme attachment to import of cytochrome c into yeast mitochondria. J. Biol. Chem. 263:15928-15937.

Eady, R.R., R. Issack, C. Kennedy, J.R. Postgate, and H. Ratcliffe. 1978. Nitrogenase synthesis in Klebsiella pneumoniae: comparison of ammonium and oxygen regulation. J. Genl. Microbiol. 104:277-285.

Englesberg, E. and J.B. Levy. 1955. Induced synthesis of tricarboxylic acid cycle enzymes as correlated with the oxidation of acetate and glucose by Pasteurella pestis. J. Bacteriol. 69:418-431.

Evans, M.J. and R.C. Scarpulla. 1988. Both upstream and intron sequence elements are required for elevated expression of the rat somatic cytochrome c gene in COS-1 cells. Mol. Cell. Biol. 8:35-41.

Falk, J.E., R.J. Porra, A. Brown, F. Moss, and H.E. Larminie. 1959. Effect of oxygen tension on haem and porphyrin biosynthesis.

Nature 184:1217-1219.

Ferreira, G.C., and H.A. Dailey. 1988. Mouse protoporphyrinogen oxidase. Biochem. J. 250:597-603.

Fox, T.D. 1986. Nuclear gene products required for translation of specific mitochondrially coded mRNAs in yeast. Trends Genet. 2:97-100.

Fox, T.D., M.C. Costanzo, C.A. Strick, and D.L. Marykwas. 1988. Yeast nuclear genes that control translation of specific mitochondrial mRNAs. Faseb J. 2:A762.

George, P. 1965. The fitness of oxygen. In Oxidases and Related Redox Systems, Vol. 1, eds, T.E. King, H.S. Mason, and M. Morrison, pp. 3-36. New York: John Wiley & Sons, Inc.

González-Halphen, D., M.A. Lindorfer, and R.A. Capaldi. 1988. Subunit arrangement in beef heart complex III. Biochemistry 27:7021-7031.

Gray, C.T., J.W.T. Wimpenny, D.E. Hughes, and M.R. Mossman. 1966a. Regulation of metabolism in facultative bacteria. I. Structural and functional changes in Escherichia coli associated with shifts between the aerobic and anaerobic states. Biochim. Biophys. Acta 117:22-32.

Gray, C.T., J.W.T. Wimpenny, and M.R. Mossman. 1966b. Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of krebs cycle enzymes

in Escherichia coli. Biochim. Biophys. Acta 117:33-41.

Guarente, L. and T. Mason. 1983. Heme regulates transcription of the CYC1 gene of S. cerevisiae via an upstream activation site. Cell 32:1279-1286.

Guarente, L., B. Lablonde, P. Gifford, and E. Alani. 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the CYC1 gene of S. cerevisiae. Cell 36:503-511.

Gussin, G.N., C.W. Ronson, and F.M. Ausubel. 1986. Regulation of nitrogen fixation genes. Ann. Rev. Genet. 20:567-591.

Haddock, B.A. and C.W. Jones. 1977. Bacterial respiration. Bacteriol. Rev. 41:47-99.

Hahn, S. and L. Guarente. 1988. Yeast HAP2 and HAP3: transcriptional activators in a heteromeric comples. Sci. 240:317-321.

Hakami, N. and D.A. Pious. 1967. Regulation of cytochrome oxidase in human cells in culture. Nature 216:1087-1090.

Hallman, M. 1971. Changes in mitochondrial respiratory chain proteins during perinatal development. Evidence of the importance of environmental oxygen tension. Biochim. Biophys. Acta 253:360-372.

Hance, A.J., E.D. Robin, L.M. Simon, S. Alexander, L.A. Herzenberg, and J. Theodore. 1980. Regulation of glycolytic enzyme activity during chronic hypoxia by changes in rate-limiting enzyme content. J. Clin. Invest. 66:1258-1264.

Hare, J.F., E. Ching, and G. Attardi. 1980. Isolation, subunit composition, and site of synthesis of human cytochrome c oxidase. Biochemistry 19:2023-2030.

Hare, J.F. and R. Hodges. 1982. Oxygen-stimulated cytochrome oxidase assembly in hepatocyte monolayer cultures. J. Cell. Physiol. 113:23-27.

Heacock, C.S. and R.M. Sutherland. 1986. Induction characteristics of oxygen regulated proteins. Int. J. Radiation Oncology Biol. Phys. 12:1287-1290.

Heady, R.E., N.J. Jacobs, and R.H. Deibel. 1964. Effect of haemin supplementation on prophyrin accumulation and catalase synthesis during anaerobic growth of Staphylococcus. Nature 203:1285-1286.

Hochachka, P.W. 1980. Living without oxygen. Cambridge: Harvard University Press.

Hodge, M.R., G. Kim, K. Singh, and M.G. Cumsky. 1989. Inverse regulation of the yeast COX 5 genes by oxygen and heme. Mol. Cell. Biol. 9:1958-1964.

Horikoshi, T., A.K. Balin, and D.M. Carter. 1986. Effect of oxygen on the growth of human epidermal keratinocytes. J. Invest. Dermatol. 86:424-427.

Iuchi, S., D.R. Kuritzkes, and E.C.C. Lin. 1986. Three classes of

Escherichia coli mutants selected for aerobic expression of fumarate reductase. J. Bacteriol. 168:1415-1421.

Iuchi, S. and E.C.C. Lin. 1988. arcA (dye), a global regulatory gene in Escherichia coli mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. 85:1888-1892.

Jacobs, N.J. and S.F. Conti. 1965. Effect of hemin on the formation of the cytochrome system of anaerobically grown Staphylococcus epidermidis. J. Bacteriol. 89:675-679.

Jones, D.P. 1988. New concepts of the molecular pathogenesis arising from hypoxia. In Oxidases and Related Redox Systems, eds., T.E. King, H.S. Mason, and M. Morrison, pp. 127-146. New York: Alan R. Liss, Inc.

Jones, D.P. and H.S. Mason. 1978. Metabolic Hypoxia: accumulation of tyrosine metabolites in hepatocytes at low pO_2. Biochem. Biophys. Res. Comm. 80:477-483.

Jones, D.P. and F.G. Kennedy. 1982. Intracellular oxygen supply during hypoxia. Am. J. Physiol. 243:C247-C253.

Jones, D.P., T.Y. Aw, and F.G. Kennedy. 1983. Isolated hepatocytes as a model for the study of cellular hypoxia. In Isolation, Characterization and Use of Hepatocytes, eds., R.A. Harris and N.W. Cornell, pp. 323-332. New York: Elsevier Sci. Publ. Co., Inc.

Kadenbach, B., J. Jarausch, R. Hartmann, and P. Merle. 1983.

Separation of mammalian cytochrome c oxidase into 13 polypeptides by a sodium dodecyl sulfate-gel electrophoretic procedure. Anal. Biochem. 129:517-521.

Kaplan, F.S., C.T. Brighton, M.J. Boytim, M.E. Selzer, V. Lee, K. Spindler, D. Silberberg, and J. Black. 1986. Enhanced survival of rat neonatal cerebral cortical neurons at subatmospheric oxygen tensions in vitro. Brain Res. 384:199-203.

Keevil, T. and H.S. Mason. 1978. Molecular oxygen in biological oxidations - an overview. Methods Enzymol. 52:3-40.

Keilin, D. 1925. On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. Proc. Roy. Soc. Lond. 98:312-339.

Klemm, D.J. and L.L. Barton. 1987. Purification and properties of protoporphyrinogen oxidase from an anaerobic bacterium, Desulfovibrio gigas. J. Bacteriol. 169:5209-5215.

Komuniecki, P.R. and L. Vanover. 1987. Biochemical changes during the aerobic-anaerobic transition in Ascaris suum larvae. Mol. Biochem. Parasitol. 22:241-248.

Koppenol, W.H. 1988. The paradox of oxygen: thermodynamics versus toxicity. In Oxidases and Related Redox Systems, eds., T.E. King, H.S. Mason, and M. Morrison, pp. 93-109. New York: Alan R. Liss, Inc.

Kranz, R.G. and R. Haselkorn. 1986. Anaerobic regulation of nitrogen-fixation genes in Rhodopseudomonas capsulata. Proc. Natl. Acad. Science. 83:6805-6809.

Kurlandzka, A. and J. Rytka. 1985. Mutants of Saccharomyces cerevisiae partially defective in the last steps of haem biosynthetic pathway: isolation and genetical characterization. J. Genl. Microbiol. 131:2909-2918.

Lascelles, J. 1964. Oxygen and the evolution of biochemical pathways. in Oxygen in the Animal Organism, ed. F. Dickens and E. Neil, pp. 657-672. New York: Pergamon Press.

Lemberg, R. and J. Barrett. 1973. The cytochromes in evolution, in Cytochromes, chapter X, pp. 484-497. London: Academic Press.

Levine, J.S. 1988. The origin and evolution of atmospheric oxygen. In Oxidases and Related Redox Systems, eds., T.E. King, H.S. Mason, and M. Morrison, pp. 111-126. New York: Alan R. Liss, Inc.

Lin, C-I. P., E.G. Gollub, and D.S. Beattie. 1982. Synthesis of the proteins of complex III of the mitochondrial respiratory chain in heme-deficient cells. Eur. J. Biochem. 128:309-313.

Loffler, M. 1989. The biosynthetic pathway of pyrimidine (deoxy)nucleotides: a sensor of oxygen tension necessary for maintaining cell proliferation? Exp. Cell Res. 182:673-680.

Lowry, C.V. and R.S. Zitomer. 1988. ROX1 encodes a heme-induced

repression factor regulating ANB1 and CYC7 of Saccharomyces cerevisiae. Mol. Cell. Biol. 8:4651-4658.

Lubbers, D.W. 1983. Fundamentals and significance of local oxygen pressure measurements and pO_2 histograms in the evaluation of oxygen supply to organs and organisms. In Determination of Tissue Oxygen Pressure in Patients, ed., A.M. Ehrly, pp. 1-13. Oxford: Pergamon Press.

Lukaszkiewicz, J. and T. Bilinski. 1979. Effect of anaerobiosis and glucose on the content of haem and its precursors in intact yeast cells. Acta Biochim. Pol. 26:161-169.

Mason, T., H. N. Little, C. VanSickle, and L. Guarente. 1983. The role of heme and oxygen in the regulation of mitochondrial cytochromes. In Mitochondria, eds., R.J. Schweyen, K. Wolf, and F. Kaudewitz, pp. 509-521. Berlin: Walter de Gruyter and Co.

Maizel, J.V. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids. In Fundamental Techniques of Virology, eds. K. Habel and N.P. Salgman, pp. 334-362. New York: Academic Press Inc.

Matsumura, T. and R.G. Thurman. 1983. Measuring rates of O_2 uptake in periportal and pericentral regions of liver lobule: stop-flow experiments with perfused liver. Am. J. Physiol. 244:G656-G659.

Mattoon, J. R., W.E. Lancashire, H.K. Sanders, E. Carvajal, D.R. Malamud, G.R.C. Braz, and A.D. Panek. 1979. Oxygen and catabolite regulation of hemoprotein biosynthesis in yeast. In Biochemical and

clinical aspects of oxygen, ed. W.S. Caughey, pp. 421-435.

McEwen, J.E., C. Ko, B. Kloeckner-Gruissem, and R.O. Poyton. 1986. Nuclear functions required for cytochrome c oxidase biogenesis in Saccharomyces cerevisiae. J. Biol. Chem. 261:11872-11879.

Mela, L., C.W. Goodwin, and L.D. Miller. 1976. In vivo control of mitochondrial enzyme concentrations and activity by oxygen. Am. J. Physiol. 231:1811-1816.

Moss, F. 1952. The influence of oxygen tension on respiration and cytochrome a formation of Escherichia coli. Aust. J. exp. Biol. med. Sci. 30:531-540.

Moss, F. 1956. Adaptation of the cytochromes of Aerobacter aerogenes in response to environmental oxygen tension. Aust. J. exp. Biol. med. Sci. 34:395-406.

Murphy, B.J., E.D. Robin, D.P. Tapper, R.J. Wong, and D.A. Clayton. 1984. Hypoxic coordinate regulation of mitochondrial enzymes in mammalian cells. Sci. 223:707-709.

Ninfa, A. J. and B. Magasanik. 1986. Covalent modification of the glnG product, NR_I, by the glnL product, NR_{II}, regulates the transcription of the glnALG operon in Escherichia coli. Proc. Natl. Acad. Sci. 83:5909-5913.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

Oren, R., A.E. Farnham, K. Saito, E. Molofsky, and M.L. Karnovsky. 1963. Metabolic patterns in three types of phagocytizing cells. J. Cell Biol. 17:487-501.

Pentland, A.P. and C.L. Marcelo. 1983. Modulation of proliferation in epidermal keratinocyte cultures by lowered oxygen tension. Exp. Cell Res. 145:31-43.

Pfeifer, K., B. Arcangioli, and L. Guarente. 1987. Yeast HAP1 activator competes with the factor RC2 for binding to the upstream activation site UAS1 of the CYC1 gene. Cell 49:9-18.

Pious, D.A. 1970. Induction of cytochromes in human cells by oxygen. Proc. Natl. Acad. Sci. 65:1001-1008.

Pollak, J.K. and C.G. Duck-Chong. 1973. Changes in rat liver mitochondria and endoplasmic reticulum during development and differentiation. Enzyme 15:139-160.

Poulson, R. and W.J. Polglase. 1974. Aerobic and anaerobic coproporphyrino-genase activities in extracts from Saccharomyces cerevisiae. J. Biol. Chem. 249:6367-6371.

Poulson, R. and W.J. Polglase. 1975. The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. J. Biol. Chem. 250:1269-1274.

Poulson, R. 1976. The enzymic conversion of protoporphyrinogen IX to

protoporphyrin IX in mammalian mitochondria. J. Biol. Chem. 251:3730-3733.

Poutre, C.G. and T.D. Fox. 1987. PET111, a Saccharomyces cerevisiae nuclear gene required for translation of the mitochondrial mRNA encoding cytochrome c oxidase subunit II. Genetics 115:637-647.

Power, S.D., M.A. Lochrie, K.A. Sevarino, T.E. Patterson, and R.O. Poyton. 1984. The nuclear-coded subunits of yeast cytochrome c oxidase. I. Fractionation of the holoenzyme into chemically pure polypeptides and the identification of the two new subunits using solvent extraction and reversed phase high performance liquid chromatography. J. Biol. Chem. 259:6564-6570.

Prezant, T., K. Pfeifer, and L. Guarente. 1987. Organization of the regulatory region of the yeast CYC7 gene: multiple factors are involved in regulation. Mol. Cell. Biol. 7:3252-3259.

Ptashne, K.A., J. Theodore, and E.D. Robin. 1983. Increased phosphofructokinase content during chronic hypoxia in cultured skeletal muscle (L8) cells. Biochim. Biophys. Acta. 763:169-174.

Riccio, P., H. Schägger, W.D. Engel, and G. von Jagow. 1977. bc1-complex from beef heart. One-step purification by hydroxyapatite chromatography in Triton X-100, polypeptide pattern and respiratory chain characteristics. Biochim. Biophys. Acta 459:250-262.

Rivett, A.J. and J.F. Hare. 1987. Enhanced degradation of oxidized glutamine synthetase in vitro and after microinjection into Hepatoma

cells. Arch. Biochem. Biophys. 259:423-430.

Ross E. and G. Schatz. 1976. Cytochrome c_1 of Baker's yeast. J. Biol. Chem. 251:1997-2004.

Robin, E.D., B.J. Murphy and J. Theodore. 1984. Coordinate regulation of glycolysis by hypoxia in mammalian cells. J. Cell. Physiol. 118:287-290.

Rowland, L.J. and J.N. Strommer. 1986. Anaerobic treatment of maize roots affects transcription of Adh1 and transcript stability. Mol. Cell. Biol. 6:3368-3372.

Sachs, M.M., M. Freeling, and R. Okimoto. 1980. The anaerobic proteins of maize. Cell 20:761-767.

Sahai, A., L.A. Cole, D.L. Clarke, and R.L. Tannen. 1989. Rocking promotes differentiated properties in LLC-PK_1 cells by improved oxygenation. Am. J. Physiol. 256:C1064-C1069.

Saltzgaber-Müller, J. and G. Schatz. 1978. Heme is necessary for the accumulation and assembly of cytochrome c oxidase subunits in Saccharomyces cerevisiae. J. Biol. Chem. 253:305-310.

Schaeffer, P. 1952. Recherches sur le métabolisme bactérien des cytochromes et des porphyrines I. Disparition partielle des cytochromes par culture anaérobie chez certaines bactéries aérobies facultatives. Biochim. Biophys. Acta. 9:261-270.

Schägger, H., Th. A. Link, W.D. Engel, and G. von Jagow. 1986. Isolation of the eleven protein subunits of the bc_1 complex from beef heart. Methods Enzymol. 126:224-237.

Schägger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDA. Anal. Biochem. 166:368-379.

Schulze, M. and G. Rödel. 1988. SCO1, a yeast nuclear gene essential for accumulation of mitochondrial cytochrome c oxidase subunit II. Mol. Gen. Genet. 211:492-498.

Schumacher, H.H. 1957. Histochemical distribution pattern of respiratory enzymes in the liver lobule. Sci. 125:501-503.

Schwerzmann, K., L.M. Cruz-Orive, R. Eggman, A. Sanger, and E.R. Weibel. 1986. Molecular architecture of the inner membrane of mitochondria from rat liver: a combined biochemical and stereological study. J. Cell Biol. 102:97-103.

Sciandra, J.J., J.R. Subjeck, and C.S. Hughes. 1984. Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation. Proc. Natl. Acad. Sci. 81:4843-4847.

Shaw, D.J. and J.R. Guest. 1982. Amplification and product identification of the fnr gene of Escherichia coli. J. Genl. Microbiol. 128:2221-2228.

Sherman, F. and J.W. Stewart. 1971. Genetics and biosynthesis of cytochrome c. Ann. Rev. Genetics. 5:257-296.

Simon, L.M., E.D. Robin, J.R. Phillips, J. Acevedo, S.G. Axline, and J. Theodore. 1977. Enzymatic basis for bioenergetic differences of alveolar versus peritoneal macrophages and enzyme regulation by molecular O_2. J. Clin. Invest. 59:443-448.

Simon, L.M., E.D. Robin, T. Raffin, J. Theodore, and W.H.J. Doublas. 1978. Bioenergetic Pattern of Isolated Type II Pneumocytes in Air and during Hypoxia. J. Clin. Invest. 61:1232-1239.

Simon, M. and G. Faye. 1984. Steps in processing of the mitochondrial cytochrome oxidase subunit I pre-mRNA affected by a nuclear mutation in yeast. Proc. Natl. Acad. Sci. 81:8-12.

Smith, M.W. and F.C. Neidhardt. 1983. Proteins induced by anaerobiosis in Eshcerichia coli. J. Bacteriol. 154:336-343.

Steffens, C.M., R. Biewald, and G. Buse. 1987. Cytochrome c oxidase is a three-copper, two-heme-A protein. Eur. J. Biochem. 164:295-300.

Stock, M.K., M.A. Asson-Batres, and J. Metcalfe. 1985. Stimulatory and persistent effect of acute hyperoxia on respiratory gas exchange of the chick embryo. Respir. Physiol. 62:217-230.

Stock, M.K. and J. Metcalfe. 1987. Modulation of growth and metabolism of the chick embryo by a brief (72-hr) change in oxygen

availability. J. Exptl. Zool. Suppl. 1:351-356.

Sugano, T., N. Oshino, and B. Chance. 1974. Mitochondrial functions under hypoxic conditions. The steady states of cytochrome c reduction and of energy metabolism. Biochim. Biophys. Acta 347:340-358.

Tait, G.H. 1972. Coproporphyrinogenase activities in extracts of Rhodopseudomonas spheroides and Chromatium Strain D. Biochem. J. 128:1159-1169.

Tappan, H. 1974. Molecular oxygen and evolution. In Molecular oxygen in biology: topics in molecular oxygen research, ed., O. Hayaishi, pp. 81-135. Amsterdam: North Holland Pub. Co.

Taylor, W.G. and R.F. Camalier. 1982. Modulation of epithelial cell proliferation in culture by dissolved oxygen. J. Cell Physiol. 111:21-27.

Tisdale, H.D. 1967. Preparation and properties of succinic-cytochrome c reductase (Complex II-III). Methods Enzymol. 10:213-214.

Trawick, J.D., R.M. Wright, and R.O. Poyton. 1989. Transcription of yeast COX6, the gene for cytochrome c oxidase subunit VI, is dependent on heme and on the HAP2 gene. J. Biol. Chem. 264:7005-7008.

Trueblood, C.E. and R.O. Poyton. 1987. Differential effectiveness of yeast cytochrome c oxidase subunit V genes results from differences in expression not function. Mol. Cell. Biol. 7:3520-3526.

Trueblood, C.E., R.M. Wright, and R.O. Poyton. 1988. Differential regulation of the two genes encoding Saccharomyces cerevisiae cytochrome c oxidase subunit V by heme and the HAP2 and REO1 genes. Mol. Cell. Biol. 8:4537-4540.

Vander, A.J., J.H. Sherman, and D.S. Luciano. 1975. Human Physiology, The Mechanisms of Body Function. New York: McGraw-Hill Book Company, p. 298.

Wallace, P.G. and A.W. Linnane. 1964. Oxygen-induced synthesis of yeast mitochondria. Nature 201:1191-1194.

Webster, D.F. and H.C. Burry. 1982. The effects of hypoxia on human skin, lung and tendon cells in vitro. Br. J. exp. Path. 63:50-55.

Webster, K.A. 1987. Regulation of glycolytic enzyme RNA transcriptional rates by oxygen availability in skeletal muscle cells. Mol. Cell. Biochem. 77:19-28.

Wharton, D.C. and A. Tzagoloff. 1967. Cytochrome oxidase from beef heart mitochondria. Methods Enzymol. 10:245-246.

Wielburski, A. and B.D. Nelson. 1984. Heme a induces assembly of rat liver cytochrome c oxidase subunits I-III in isolated mitochondria. FEBS Lett. 177:291-294.

Williams, J.N. 1964. A method for the simultaneous quantitative estimation of cytochromes a, b, c_1 and c in mitochondria. Arch. Biochem. Biophys. 107:537-543.

Wilson, G., R. Hodges, and J.F. Hare. 1981. Site of synthesis of the mitochondrial cytochromes in hepatocyte cultures. J. Biol. Chem. 256:5197-5203.

Wilson, J.E. 1972. The relationship between glycolytic and mitochondrial enzymes in the developing rat brain. J. Neurochem. 19:223-227.

Winkelman, J.W. and D.P. Clark. 1986. Anaerobically induced genes of Escherichia coli. J. Bacteriol. 167:362-367.

Wölfle, D. and K. Jungermann. 1985. Long-term effects of physiological oxygen concentrations on glycolysis and gluconeogenesis in hepatocyte cultures. Eur. J. Biochem. 151:299-303.

Woodrow, G. and G. Schatz. 1979. The role of oxygen in the biosynthesis of cytochrome c oxidase of yeast mitochondria. J. Biol. Chem. 254:6088-6093.

Woods, J.S. 1977. Heme regulation of cytochrome oxidase synthesis in fetal rat liver. Mol. Pharmacol. 13:50-59.

Yamamoto, N. and M.L. Droffner. 1985. Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe Salmonella typhimurium. Proc. Natl. Acad. Sci. 82:2077-2081.

Zagorec, M., J-M. Buhler, I. Treich, T. Keng, L. Guarente, and R.

Labbe-Bois. 1988. Isolation, sequence, and regulation by oxygen of the yeast HEM13 gene coding for coproporphyrinogen oxidase. J. Biol. Chem. 263:9718-9724.

Zagorec, M. and R. Labbe-Bois. 1986. Negative control of yeast coproporphyrinogen oxidase synthesis by heme and oxygen. J. Biol. Chem. 261:2506-2509.

Zeviani, M., S.Sakoda, A.A. Sherbany, H. Nakase, R.Rizzuto, C.E. Samitt, S. DiMauro, and E.A. Schon. 1988. Sequence of cDNAs encoding subunit Vb of human and bovine cytochrome c oxidase. Gene 65:1-11.

Zhu, Y.S. and J.E. Hearst. 1986. Regulation of expression of genes for light-harvesting antenna proteins LH-1 and LH-II; reaction center polypeptides RC-L, RC-M, and RC-H; and enzymes of bacteriochlorophyll and carotenoid biosynthesis in Rhodobacter capsulatus by light and oxygen. Proc. Natl. Acad. Sci. 83:7613-7617.

Zhu, Y.S. and J.E. Hearst. 1988. Transcription of oxygen-regulated photosynthetic genes requires DNA gyrase in Rhodobacter capsulatus. Proc. Natl. Acad. Sci. 85:4209-4213.

Zitomer, R.S., J.W. Sellers, D.W. McCarter, G.A. Hastings, P. Wick and C.V. Lowry. 1987. Elements involved in oxygen regulation of the Saccharomyces cerevisiae CYC7 gene. Mol. Cell. Biol. 7:2212-2220.

A D D E N D U M

Summaries of Radioactive Counts Present in Identified Bands Excised from Sodium Dodecyl Sulfate Polyacrylamide Gels

The data in Tables I-V were obtained from densitometric analysis of fluorographs or from protein bands cut from SDS polyacrylamide gels. The corresponding fluorographs were used to locate bands of interest on the dried gels. The bands were cut out and dissolved as described in Chapter 3, Experimental Methods, and the radioactive counts were determined with a scintillation counter. Fluorographs were scanned with a Bio-Rad densitometer and the areas under specified peaks were integrated with a computer assisted software program.

Table I. Synthesis of the mitochondrial translation products, cytochrome oxidase subunits I, II, and III and apocytochrome b. Cells were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 1-2h, and harvested immediately after the pulse. Cell membrane lysates were prepared from 3 flasks of cells in each group. Samples applied to the gel wells were normalized to cell number in the low oxygen sample. The percentages reported in Tables II and V, Chapter Five, (for the corresponding experiments) were calculated from the fluorometric data below. Experiment 1, Tables II and V, Chapter Five, was analyzed by densitometry only.

Expt.	Subunit	Low O_2		Control O_2	
		Counts*	Area@	Counts	Area
1	I	298	1.84	513	3.35
	II	218	0.76	249	1.00
	III	139	0.42	208	0.61
	Apo b	201	0.95	240	1.09
3	I	1142	3.34	2505	5.56
	II	882	1.84	1180	1.89
	III	759	1.85	1196	2.21
	Apo b	500	1.52	622	1.85
4	I	2856	4.64	2900	4.64
	II	1198	2.29	1333	2.28
	III	1378	2.32	1873	2.73
	Apo b	765	1.34	952	1.86

*Radioactivity (CPM) in protein band extracted from the dried gel

@Integrated area under specified peak on the fluorograph (mm^2)

Table II. Assembly of cytochrome b. Cells were grown in low or control oxygen for 24h; pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate test gas for 2h; and chased in methionine-supplemented, label free medium in the presence of low or control oxygen for 2h. Cell membrane lysates, prepared from 3 flasks in each group, were immunoprecipitated with antisera directed against a core protein and cytochrome c_1 of the cytochrome b-c_1 holoenzyme complex. Samples applied to the gel wells were normalized to cell number in the low oxygen sample. Lanes 2 and 3 of Fig. 6, Chapter Five, correspond to the experiment below.

Subunit	Low O_2		Control O_2	
	Counts	Area	Counts	Area
Cyt b	88	0.66	309	2.22

Table III. Assembly of the mitochondrially translated subunits of cytochrome oxidase. Cells were grown for 24h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate test gas for 2h; and chased in methionine-supplemented, label free medium in the presence of low or control oxygen for 2.5h. Cell membrane lysates were prepared from three flasks per group in experiment 1 and one flask per group in experiment 2. The lysates were immunoprecipitated with antisera directed against the holoenzyme of cytochrome oxidase. Samples were normalized to cell number in the low oxygen sample. Percentage data for experiments 2 and 3 (+ CHI) reported in Table I, Chapter Five, were calculated from the fluorometric data below.

Expt.	Subunit	Low O_2		Control O_2	
		Counts	Area	Counts	Area
2	I	103	0.79	741	8.11
	II	113	1.47	455	6.17
	III	47	0.58	138	2.01
3	I	453	3.12	791	8.03
	II	584	5.19	947	8.48
	III	207	1.95	317	3.41

Table IV. Assembly of the mitochondrially-translated and the cytoplasmically translated subunits of cytochrome oxidase. Cells were labeled, chased, and prepared as described in Table III with the exception that cycloheximide was not included in the labeling medium. Samples applied to the gel wells were normalized to TCA precipitable counts present in the low oxygen post-membrane supernate. Lanes 3 and 4 of the fluorograph shown in Fig. 3, Chapter Five, correspond to experiment 2; lanes 8 and 9 of Fig. 3, Chapter Five, correspond to experiment 3.

Expt.	Subunit	Low O_2		Control O_2	
		Counts	Area	Counts	Area
2	I	161	0.28	419	3.36
	II	155	1.74	401	5.09
	III	144	1.24	232	2.84
	IV	272	3.36	415	5.28
	V	188	1.43	249	2.29
	VI	65	0.82	101	1.42
3	I	175	0.50	294	1.62
	II	208	2.05	369	3.79
	III	140	0.35	211	0.71
	IV	82	0.53	96	0.53
	V	251	1.87	257	2.08
	VI	198	1.25	216	1.16
	VII	86	0.66	90	0.58
	VIII	31	0.38	40	0.33

Note: The data for experiment 2, above, do not correspond to data reported for experiment 2 (-CHI) in Table I, Chapter Five; the latter data are from samples normalized to cell counts rather than radioactivity. Radioactivity in individual protein bands were not determined for the experiment 2 data in Table I, Chapter Five. As

there was little effect on cell-specific radioactivity in experiment 3 (-CHI, Table I, Chapter Five), normalizing these samples to radioactivity was the same as normalizing them to cell number. Thus, the percentages for this experiment were calculated from the fluorographic data for experiment 3 above. Data for experiment 1, Table I, Chapter Five, were analyzed by densitometry only.

Table V. Degradation of the mitochondrial translation products, cytochrome oxidase subunits I, II, and III, and apocytochrome *b*. Cells were grown for 15h in low or control oxygen, pulse-labeled with [^{35}S] methionine in the presence of cycloheximide and the appropriate gas for 2h; and chased for 24h in methionine-supplemented label free medium in the presence of low and control oxygen. Cell membrane lysates were prepared from 3 flasks in each group. Samples applied to the gel wells were normalized to cell number in the low oxygen sample at the beginning of the chase. The percentages reported in Table IV, Chapter Five, were calculated from the radioactivity data below. The fluorograph from this experiment was not analyzed by densitometry.

Subunit	Low O_2	Control O_2
COX I	451	537
COX II	305	315
COX III	210	227
Apo b	103	105