Improved Techniques for Identification and Separation of the Beef Heart Mitochondrial Na^+/Ca^{2+} Antiporter

Baoyu Lin
Master of Medicine, Nanjing Railway Medical College, China, 1989
Bachelor of Medicine, Nantong Medical College, China, 1982

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Keith D. Garlid, Advisor
Professor

Xiaocheng Sun
Research Assistant Professor

Gebre Woldegiorgis
Associate Professor

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ABBREVIATIONS

ADP	adenosine diphosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCECF	2-7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein
Ca^{2+}	Calcium cation
CHAPS	3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate
CIP	calf-intestinal alkaline phosphatase
CMC	critical micelle concentration
DTT	dithiothreitol
DEAE	*O*-diethylaminoethyl
ECL	enhanced chemi-luminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-N,N-tetra-acid
HEPES	N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)
HA	hydroxylapatite
ICDH	isocitrate dehydrogenase
IPTG	isopropyl-β-D-thiogalactoside
KGDH	α-ketoglutarate dehydrogenase
LB	luria-bertiani
Na^{+}	Sodium cation
NAD	nicotinamide adenine dinucleotide
Ni-NTA	nickel-nitrilotriacetic acid
PDH	pyruvate dehydrogenase
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride

SBFI	sodium-binding benzofuran isophthalate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMP	submitochondrial particle
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TCA	trichloroacetic acid
TEA^+	tetraethylammonium cation
TES	N-tris(hydroxymethyl)methy-2-aminoethenesulfonic acid
TH	mitochondrial nicotinamide nucleotide transhydrogenase
TPP^+	tetraphenylphosphonium

Abstract

Improved Techniques for Identification and Separation of the Beef Heart Mitochondrial Na^+/Ca^{2+} Antiporter

Baoyu Lin, M.S.

Supervising Professor: Keith D. Garlid

The Na^+/Ca^{2+} antiporter in plasma membranes and the membranes of organelles such as the mitochondrion plays a very important role in the regulation of Ca^{2+}. In turn, this affects cell metabolism and function, coordinates cellular energy demands and mitochondrial ATP production, and may be a critical factor in various pathological conditions. Mitochondrial Na^+/Ca^{2+} antiporter activity, first identified in the laboratory of Dr. Keith Garlid, is associated with a 110-kDa mitochondrial inner membrane protein capable of Na^+-dependent Ca^{2+} and Ca^{2+}-dependent Na^+ transport (W. Li et al., 1992). Although positive clones isolated from a bovine heart λgt11 cDNA library by screening with polyclonal antibodies against this 110-kDa protein were subsequently shown to be a mitochondrial nicotinamide nucleotide transhydrogenase (TH), success in expressing TH in *E. coli* provided a promising tool with which to separate the Na^+/Ca^{2+} antiporter protein from the TH contaminant by immuno-affinity chromatography (Cheng, 1995). As there is strong evidence that there are only two proteins in this 110-kDa band (the TH protein and the Na^+/Ca^{2+} antiporter; see Cheng, 1995), the work reported here focused on optimizing the conditions for immuno-affinity purification of these two proteins.

Based on previous work in our laboratory, the purification of the Na^+/Ca^{2+} antiporter was improved to obtain a pure protein for internal peptide sequencing. This was achieved by (1) enrichment of the 110-kDa protein sample from a DEAE-cellulose column without concentrating the detergent by using a hydroxylapatite

column; (2) single-band purification of the 110-kDa protein by electrophoresis and electro-elution prior to immuno-affinity column purification; (3) optimization of the conditions for the affinity chromatography with respect to the preparation of both the protein sample and the column itself. Based on western blots of the immuno-affinity chromatography results, the purity of the Na^+/Ca^{2+} protein in the flow-through fraction was significantly improved. Although some questions and problems remain, the purification of the Na^+/Ca^{2+} antiporter has been achieved and improved by the work reported here.

CHAPTER 1
INTRODUCTION

1.1 Ca^{2+} Function in Cells

1.1.1 Intracellular messengers and muscle contraction

The calcium ion plays an important role in the regulation of muscle contraction in skeletal and smooth muscles as well as those of the myocardium. In resting muscle tissue, Ca^{2+} is pumped into the sarcoplasmic reticulum (SR), so that the Ca^{2+} concentration around the muscle fiber is very low. Nerve impulse excitation of the SR membrane leads to a sudden release of large amounts of Ca^{2+}, triggering muscle contraction through troponin and tropomyosin (Stryer, 1988; Alberts et al., 1989).

Ca^{2+} is also an important intracellular messenger in many signal-transduction pathways. All cells and some organelles, such as mitochondria, have a transport system for the extrusion of Ca^{2+}. The cytosolic Ca^{2+} concentration can be rapidly increased for signaling purposes by transient opening of Ca^{2+} channels in the plasma or intracellular membranes.

1.1.2 Ca^{2+} as a secondary messenger in mitochondria

1.1.2.1 ***Mitochondrial and cytosolic Ca^{2+} regulation.*** As a secondary messenger system in cellular signal transduction, Ca^{2+} has been well characterized (Rasmussen & Barrett, 1984; Rasmussen & Rasmussen, 1990). The regulation of cytosolic Ca^{2+} has long been regarded as essential.

As individual mitochondria are able to accumulate massive amounts of Ca^{2+}, it first appeared that mitochondria might regulate cytosolic Ca^{2+} by accumulation or release in response to cellular signals. This idea was particularly prevalent in earlier

discussions of hepatocytes (Scarpa & Graziotti, 1973; Carafoli et al., 1976; Nicholls, 1978; Akerman, 1980; Nicholls & Akerman, 1982; Joseph & Williamson, 1983; Nedergaard, 1984; Rasmussen & Barrett, 1984). However, this concept was seriously questioned by subsequent studies demonstrating that extravesicular Ca^{2+} levels were determined predominantly by the endoplasmic reticulum rather than mitochondria (Becker et al., 1980; Streb & Schultz, 1983). Current data suggest that mitochondria probably play a minor role in regulating cytosolic Ca^{2+} levels, so that the relationship between Ca^{2+} signals and mitochondria needs further re-evaluation.

As a cellular messenger, Ca^{2+} is known to trigger a cascade of cellular responses, and it is quite conceivable that it affects mitochondrial function as well. Indeed, Ca^{2+} has been reported to activate key mitochondrial matrix enzymes related to mitochondrial ATP production, the vital role of mitochondria in eukaryotic cells.

1.1.2.2 ***Matrix enzymes regulated by Ca^{2+}.*** Potential targets for Ca^{2+} are those mitochondrial enzymes involved in ATP production, namely enzymes of the tricarboxylate cycle. The tricarboxylic acid cycle is the final common pathway for the oxidation of "fuel" molecules, amino acids, fatty acids, and carbohydrates. The cycle also provides intermediates for biosyntheses (Stryer, 1988). In heart and other mammalian tissues, there are three mitochondrial enzymes which have a regulatory function in the citric acid cycle: pyruvate, isocitrate and α-ketoglutarate dehydrogenases. Each of these enzymes is activated by an increase in intramitochondrial free Ca^{2+} concentration within the range of 0.1–10 μM (Denton et al., 1972, 1978; McCormack & Denton, 1979) (Figures 1.1 and 1.2).

Additionally, pyrophosphatase is affected by Ca^{2+}, leading to increases in mitochondrial volume and adenine nucleotide content, which in turn stimulate oxidative phosphorylation and NADP utilization (Davidson & Halestrap, 1987, 1988, 1989). The ATPase–ATP synthetase complex in the mitochondrial inner membrane is activated by a Ca^{2+}-dependent dissociation of an inhibitory subunit (Yamada et al., 1980; Yamada & Huzel, 1988). By releasing the inhibitor peptide from ATPase, Ca^{2+} can stimulate ATPase activity.

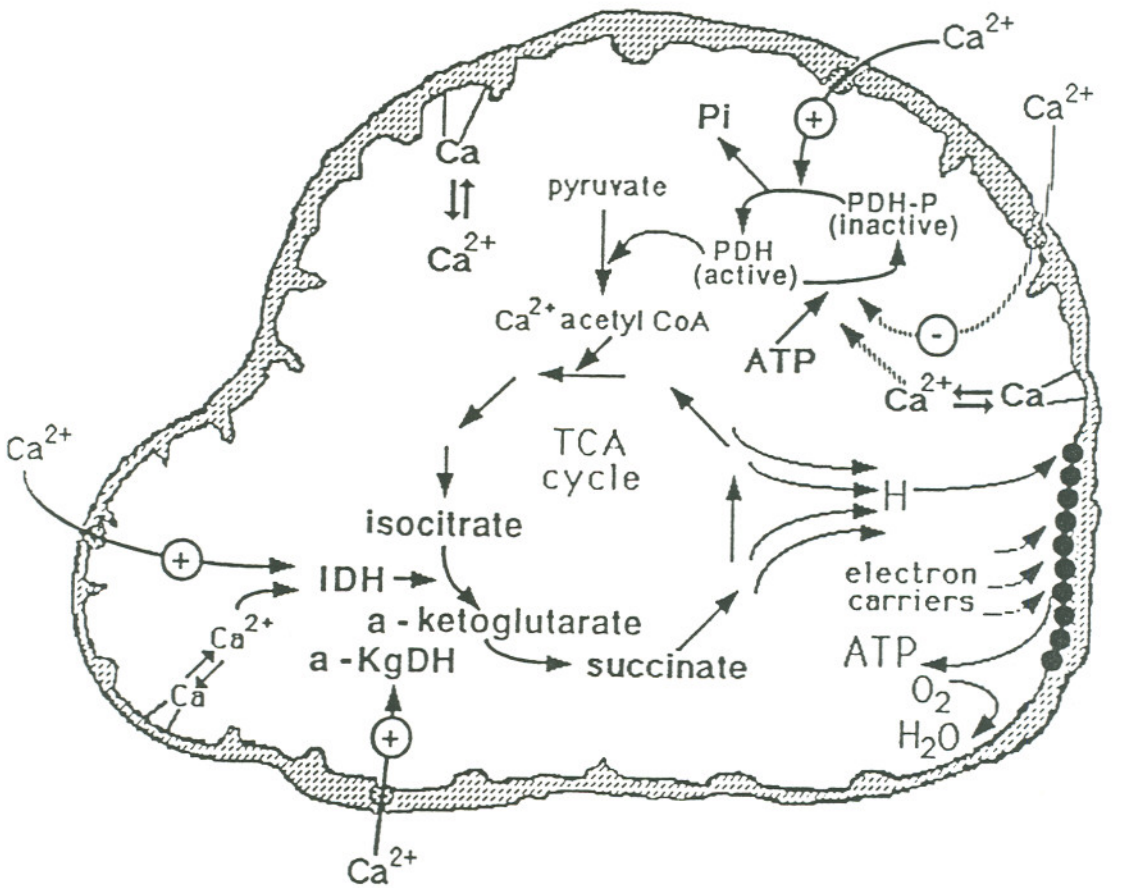

Figure 1.1 Regulation of matrix enzymes by Ca^{2+} (from Denton et al., 1972, 1978). PDH, pyruvic acid dehydrogenase; IDH, NAD-linked isocitric acid dehydrogenase; α-KgDH, α-ketoglutaric acid dehydrogenase (Carafoli, 1988).

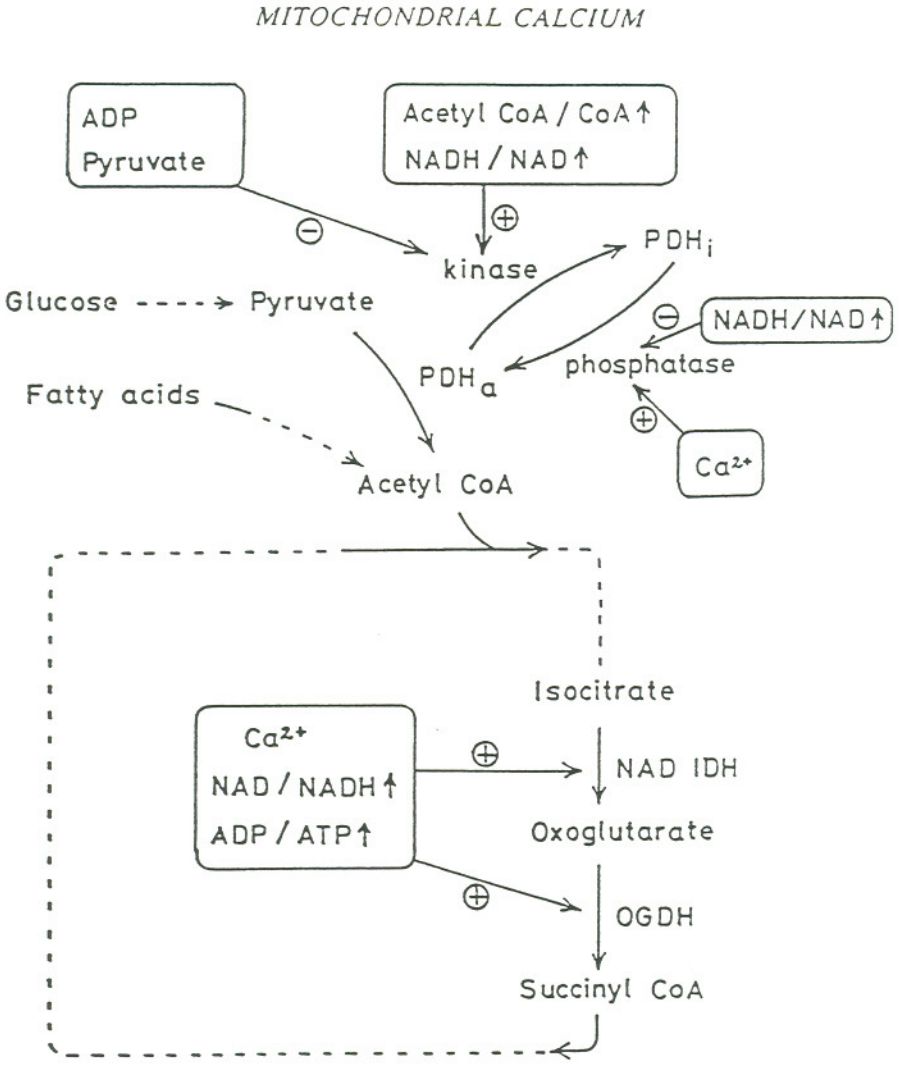

Figure 1.2 Regulation of mitochondrial oxidations by Ca^{2+} and other factors. The scheme shows entry of acetyl CoA, derived from glucose and fatty acids, into the tricarboxylic acid cycle. Pyruvate dehydrogenase (PDH) is converted to an inactive form (PDH_i) by phosphorylation catalyzed by PDH kinase; conversion to the active form (PDH_a) is accomplished by dephosphorylation catalyzed by PDH phosphatase. PDH kinase, PDH phosphatase, and the two tricarboxylic acid cycle enzymes, NAD-linked isocitrate dehydrogenase (NAD IDH) and oxoglutarate dehydrogenase (OGDH), are regulated as indicated by Ca^{2+}, ADP, and pyruvate and by increased ratios (denoted by ↑) of NAD/NADH, ADP/ATP, and acetyl CoA/CoA. Enzyme activation is denoted by ⊕, and inhibition is denoted by ⊖ (Crompton, 1990).

These effects probably represent the most important aspects of calcium signaling with respect to mitochondrial function. Significantly, all three processes seem to be modulated at matrix-free calcium concentrations of $<1\ \mu M$.

1.1.2.3 ***A new concept: Matrix Ca^{2+} regulates mitochondrial ATP production.*** The phosphorylation potential, long viewed as the key regulator of mitochondrial ATP production, has been observed to be quite stable under many experimental conditions in which cellular energy demand changes (Neely et al., 1972; Balaban et al., 1986; Unitt et al., 1989). Hansford (1985) and Denton and McCormack (1985) found that surges of cytosolic calcium levels were readily relayed into the matrix by a mitochondrial Ca^{2+} transport system, where it stimulated ATP production by modulating matrix enzymatic activity. They proposed that the mitochondrial Ca^{2+} transport system regulates matrix Ca^{2+} activity, rather than cytosolic Ca^{2+} levels, so that changes in cytosolic Ca^{2+}, which are correlated with cellular energy demands, thus signal mitochondria to alter ATP production. This view of the regulation of mitochondrial ATP production by Ca^{2+} offers an advantage over the classic regulator, phosphorylation potential, in as much as Ca^{2+}-induced cellular events, such as muscle contraction and hormone secretion, substantially increase ATP requirements.

Current evidence shows that the cellular energetic state is primarily determined and reflected by matrix Ca^{2+} activity. The feedback mechanism controlling the ADP:ATP ratio appears poised to maintain a relatively stable phosphorylation potential and stable energetic state. Cellular energy demand is transmitted to mitochondria via a Ca^{2+} signal; the resulting energy state is correlated with a phosphorylation potential which corresponds to matrix Ca^{2+} activity. Thus, when regulation of mitochondrial ATP production via the phosphorylation potential maintains a steady state of mitochondrial ATP production, a new steady-state level can be established by a change of matrix Ca^{2+} activity. Hence, the regulation of cellular energy states can be achieved by regulation of the mitochondrial Ca^{2+} cycle. This calcium regulation of matrix enzymes affecting mitochondrial ATP production is consistent with calcium's role as a cellular messenger.

1.1.3 Other biological functions of calcium

In the mammalian nerve cell, surges of free Ca^{2+} act as intracellular messengers to trigger the release of neurotransmitters at a rate that increases vary sharply with free Ca^{2+} concentration. Experiments by Hepler & Callaham (1987) suggested that the initiation of anaphase involved an increase in cytosolic Ca^{2+}. They proposed the possibility of a spindle-associated vesicle release of Ca^{2+} to initiate anaphase analogous to the release of Ca^{2+} by the SR to initiate skeletal muscle contraction. Ca^{2+}-dependent systems play a crucial part in the adhesion of cells. In cell culture media, it is known that if the concentration of Ca^{2+} is kept abnormally low, the cell–cell adhesion system cannot operate (Hennings & Holbrook, 1983). There is strong evidence that a transient increase in cytosolic Ca^{2+} concentration, which propagates as a wave across the egg from the site of sperm fusion, helps to initiate zygote development (Eisen & Reynolds, 1985). The permeability of the gap junction is also regulated by Ca^{2+}, being rapidly and reversibly decreased by increases in cytosolic concentration of free Ca^{2+}. Thus when a cell dies or is damaged, its membrane becomes leaky and ions (such as Ca^{2+} and Na^{+}) move into the cell while valuable metabolites leak out. Were the damaged cell to remain coupled to its healthy neighbors, these too would suffer a dangerous disturbance of their internal chemistry. The influx of Ca^{2+} into the dysfunctional cell, by closing the gap junction channels, effectively isolates it and prevents the damage from spreading (Rose & Loewenstein, 1975).

1.2 Plasma Membrane Control of Cell Calcium Concentration: A Signaling Mechanism

The very low Ca^{2+} permeability of the plasma membrane insulates the intracellular milieu from the high concentrations of Ca^{2+} in the extracellular spaces. In most eukaryotic cells, the inwardly directed gradient of Ca^{2+} is on the order of 10^4. The signaling function requires that the free Ca^{2+} concentration surrounding the cytosolic targets change rapidly, reversibly, and significantly. The large Ca^{2+}

gradient ensures that even a minor (controlled) increase in Ca^{2+} permeability of the plasma membrane leads to rapid Ca^{2+} influx and significant changes in the intracellular free Ca^{2+} concentration.

In addition to being the barrier through which the "trigger" Ca^{2+} penetrates into cells, the plasma membrane is by definition the final controller of the Ca^{2+} balance between the intra- and extracellular spaces. Its Ca^{2+} importing and exporting systems are poised in such a way to produce a set point which results in the 10,000-fold difference in the ionized Ca^{2+} concentration that normally exists between intra- and extracellular spaces in adult cells (Carafoli & Longoni, 1987).

Eukaryotic cells possess several Ca^{2+} transport systems in the plasma membrane as well as in the membrane of their organelles. These systems operate with differing kinetic properties, satisfying the various requirements of the functional cycles of the cells. Such demands may be for rapid high-affinity regulation of Ca^{2+} or for less rapid lower-affinity regulation. In a typical eukaryotic cell, there are at least seven Ca^{2+} transporting systems intrinsic to membranes, corresponding to four transport models: ATPases, exchangers (antiporter), channels, and electrophoretic uniporters (see Figure 1.3). High-affinity Ca^{2+} regulation depends on the work of ATPases, the only systems capable of high-affinity interaction with Ca^{2+}, while lower-affinity regulation appears to be associated with the other three transporting models (Carafoli, 1988).

The long-term, algebraic sum of the three Ca^{2+} transporting operations under physiological conditions must, of necessity, be zero, because Ca^{2+} overload or depletion does not normally occur in cells. The overall operation of the two Ca^{2+} exporting systems will thus offset the importing function of the Ca^{2+} channel. Ca^{2+} ATPase operates as a system designed to continuously eject Ca^{2+} from cells. Its high affinity for Ca^{2+} permits it to function in the submicromolar concentrations present in most cytosols, although its total transport capacity is low. In contrast, the Na^{+}/Ca^{2+} exchanger, with its low Ca^{2+} affinity, works efficiently only when Ca^{2+} concentration in the cytosol increases to higher levels (Carafoli & Longoni, 1987).

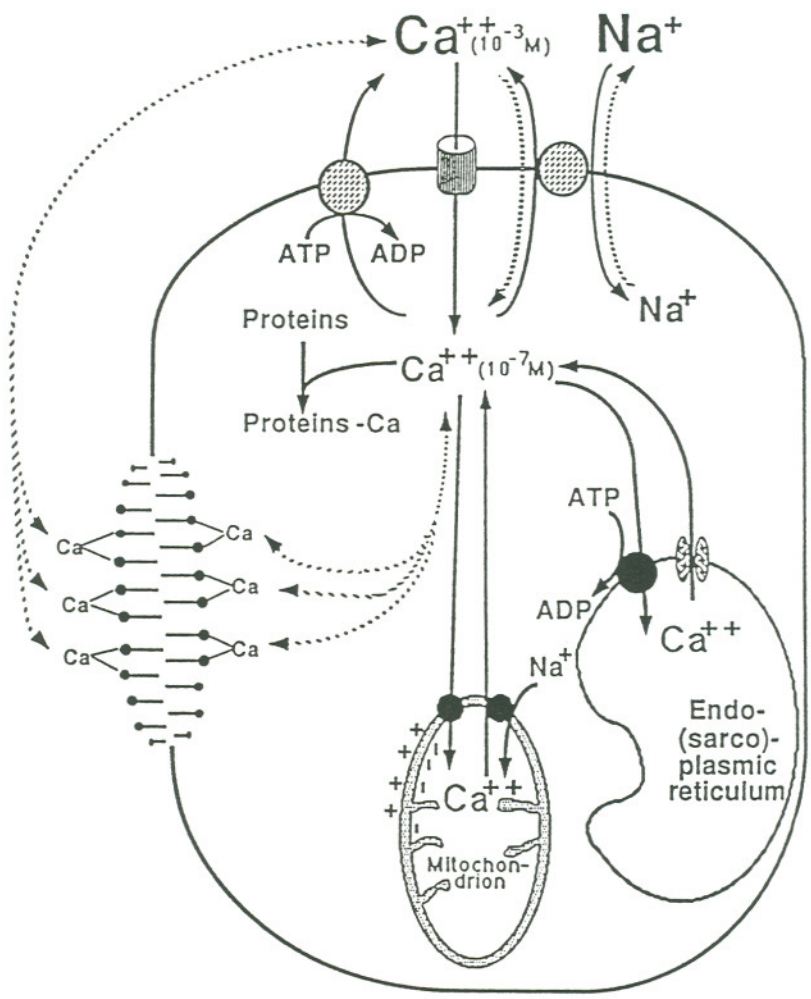

Figure 1.3 Ca^{2+} transporting systems in eukaryotic cells. The figure shows seven transporting systems: three in the plasma membrane (Ca^{2+}-ATPase, Na^+/Ca^{2+} exchanger, and Ca^{2+} channel), two in the endo(sarco)plasmic reticulum (Ca^{2+}-ATPase and a release channel), and two in the inner membrane of mitochondria (an electrophoretic uptake uniporter and a Ca^{2+}-releasing Na^+/Ca^{2+} exchanger). The systems in the Golgi membranes and in the lysosomes have not been included as they are still insufficiently characterized. The soluble Ca^{2+}-binding proteins of the cytosol and the acidic phospholipids of the plasma membrane have been included in the figure as participants in the Ca^{2+}-buffering function (Carafoli, 1988).

1.3 The Na^+/Ca^{2+} Antiporter in Plasma and Mitochondrial Membranes

The Na^+/Ca^{2+} antiporter is one of two Ca^{2+} exporting systems of the plasma membrane (although under some conditions it can also mediate the influx of Ca^{2+}). It is a large-capacity low-affinity system which is particularly active in excitable tissues. The Na^+/Ca^{2+} antiporter is also critical in normal cell function and is distributed broadly in different tissues, cells, and organelles. This exchanger is not identical in different cell types (Reuter, 1991) and it is clear that the function of the Na^+/Ca^{2+} antiporter is different in different tissues (Schulze et al., 1993). Experimental evidence [summarized by DiPolo (1989)], suggesting the presence of a Na^+/Ca^{2+} exchange-mediated mechanism, came from cardiac muscle cells as early as 1957. The actual discovery of a Na^+/Ca^{2+} antiporter emerged in the late 1960s from the work of Baker et al. (1967) and Reuter and Seitz (1968). These two independent groups, working on the squid giant axon and cardiac muscle, respectively, found that the efflux of Ca^{2+} was highly dependent on the presence of external Na^+. Furthermore, the influx of Ca^{2+} was enhanced by removal of the external Na^+ or by increasing the internal Na^+, thus indicating the presence of a reversible carrier system able to couple the movement of Na^+ for Ca^{2+} across the membrane in either direction.

1.3.1 Distribution of Na^+/Ca^{2+} in tissues

The Na^+/Ca^{2+} exchanger has been studied in a wide variety of preparations, including cardiac muscle, brain synaptosomes, and plasma membrane vesicles from different organisms. Although the exchanger seems to be most active in heart and nerve cells, it is also found in non-excitable tissues such as epithelial cells, endocrine tissues, bone cells, and dog and ferret erythrocytes. Some experiments [summarized by Saermark (1989)] have shown that Na^+/Ca^{2+} exchange exists in secretory vesicles and thus may play a role in Ca^{2+} buffering in secretory cells. Indeed, the Na^+/Ca^{2+} exchange system is present in most all types of mammalian smooth (Blaustein, 1989) and skeletal muscle, as well as renal, pancreatic acinar, and pituitary neurohypophysis cells, and platelets (Rahamimoff, 1989).

1.3.2 Functional importance of the Na^+/Ca^{2+} antiporter

1.3.2.1 ***Function of the Na^+/Ca^{2+} antiporter in sarcolemma.*** It is now accepted that in sarcolemma the Na^+/Ca^{2+} antiporter exchanges three Na^+ for one Ca^{2+} ion and is therefore electrogenic. The fact that the antiporter is charged means that its transport is influenced by the trans-sarcolemmal potential. Bridge et al. (1990), using voltage-clamp procedures and rapid solution changes in isolated ventricular cells, demonstrated that all Ca^{2+} entering the cell via the Ca^{2+} channel could be removed via the Na^+/Ca^{2+} antiporter during the course of a single contraction cycle. Although the antiporter functions predominantly in the Ca^{2+} efflux mode, a relatively small increase in the internal Na^+ concentration causes reversal of the next fluxes. External Na^+-dependent Ca^{2+} efflux for inside-out vesicles (corresponding to internal Na^+-dependent Ca^{2+} influx in the intact cell) has a strong dependence on Na^+ concentration between 8 and 12 mM (Philipson & Nishimoto, 1982), a reasonable range for internal $[Na^+]$ in the intact cell (Lee et al., 1980; Cohn et al., 1982). The inhibition of sarcolemmal Na^+/K^+ ATPase by digitalis glycosides results in the elevation of internal $[Na^+]$ and a significant increase in contractile force. It is now agreed that increased internal $[Na^+]$ causes net Ca^{2+} uptake by the cell through its effect on the Na^+/Ca^{2+} antiporter. Leblanc and Hume (1990) proposed another interesting role for this antiporter, suggesting that a transient rise in internal $[Na^+]$ occurs in the diffusion-restricted region near the intracellular opening of the Na^+ channel during depolarization. This increase would cause a transient net influx of Ca^{2+} in the region of the SR feet by reversal of the Na^+/Ca^{2+}antiporter. The net result is a Ca^{2+}-induced Ca^{2+} release and consequent contractile activation.

1.3.2.2 ***Function of the Na^+/Ca^{2+} antiporter in the SR.*** Although most investigations have so far failed to demonstrate the presence of a Na^+/Ca^{2+} antiporter in the SR (Carafoli, 1988; Langer, 1992; Schulze et al., 1993), a few researchers inferred its existence as a mechanism which can import Ca^{2+} into the cell during the action potential (internal Ca^{2+} transient) and extrude Ca^{2+} from the cell during the resting period (diastole) when internal $[Ca^{2+}]$ is high. Sipido and Wier (1991) summarized these results: the direct substantive contribution of Ca^{2+} influx via the

Na^+/Ca^{2+} antiporter to the $[Ca^{2+}]_i$-transient during excitation-contraction coupling has been found so far only in guinea pig cells. On the other hand, when $[Na^+]_i$ is elevated and the membrane is strongly depolarized, slow contraction can be activated by Ca^{2+} entering via the Na^+/Ca^{2+} exchanger. Some researchers have also reported that the ventricular myocyte SR is functionally associated with Ca^{2+} efflux through the Na^+/Ca^{2+} antiporter.

1.3.2.3 ***Function of the Na^+/Ca^{2+} antiporter in mitochondria.*** In a comprehensive review of Ca^{2+} interaction in mitochondria, Crompton (1990) noted that it has long been recognized that mitochondria transport Ca^{2+} and account for approximately 20% of total cellular Ca^{2+}. Yet Ca^{2+} transport is neither a primary nor essential role of mitochondria. Most probably, Ca^{2+} transport is directed toward regulation of the various intramitochondrial enzymes responsible for control of oxidative metabolism and ATP production by this organelle.

1.3.2.3.1 Distribution of the mitochondrial Na^+/Ca^{2+} antiporter in different tissues. Na^+-dependent Ca^{2+} efflux was initially demonstrated in cardiac mitochondria (Carafoli et al., 1974; Crompton et al., 1977). Crompton and Heid (1978) found Na^+/Ca^{2+} exchange in mitochondria from heart, brain, adrenal cortex, parotid gland, and skeletal muscle, but failed to find such activity in mitochondria from liver, kidney, lung, uterus muscle, or ileum muscle. Subsequently, other laboratories did succeed in demonstrating Na^+-dependent Ca^{2+} efflux in mitochondria from kidney and lung (Haworth et al., 1980; Heffron & Harris, 1981; Nedergaard, 1984; Rizzuto et al., 1987).

Data from both isolated mitochondria and the purified Na^+/Ca^{2+} antiporter support the view that the Na^+/Ca^{2+} antiporter exists in the mitochondria of both excitable and non-excitable tissues. This broad distribution supports the concept of the Na^+/Ca^{2+} antiporter's universal regulatory role of Ca^{2+} in mitochondrial function.

1.3.2.3.2 The mitochondrial Ca^{2+} cycle. An elaborate calcium transport system in the mitochondrial inner membrane has been uncovered over the past two decades. This system requires metabolic energy expenditure and results in

calcium cycling across the inner membrane. Mitochondrial respiration, the Na^+/H^+ antiporter, Ca^{2+} uniporter, and Na^+/Ca^{2+} antiporter, together comprise four major components of the mitochondrial calcium cycle, as diagrammed in Figure 1.4.

Mitochondria generate and maintain a proton gradient across the inner membrane by respiration, as postulated in Mitchell's chemosmotic theory (Mitchell & Moyle, 1969). The proton gradient, the central motif of mitochondrial function, is the result of electron energy transfer from the oxidation of substrates. Mitochondrial metabolism, functional regulation, volume homeostasis, and pH regulation, depend entirely on this proton gradient. With respect to the Ca^{2+} cycle, the proton gradient provides the ultimate driving force needed to maintain proper matrix Ca^{2+} activity. The net effect of the mitochondrial calcium transport system is a futile calcium cycle across the inner membrane at the expense of respiration energy. This energy expenditure is the cost of regulating matrix Ca^{2+} activity and mitochondrial metabolism.

The carriers directly involved in Ca^{2+} transport are the Na^+/Ca^{2+} antiporter, which extrudes Ca^{2+} by depleting the Na^+ gradient built up by the Na^+/H^+ antiporter, and the calcium uniporter, which mediates rapid calcium uptake into the matrix. The importance of the Na^+/H^+ antiporter to mitochondrial calcium transport has to do with its ability to maintain an inward sodium gradient. It is generally accepted that the Na^+/H^+ antiporter maintains a sodium gradient that is in equilibrium with respect to the proton gradient. It is this Na^+ gradient that provides the driving force to extrude Ca^{2+} via the Na^+/Ca^{2+} antiporter.

The calcium uniporter has been characterized as a high-capacity Ca^{2+} uptake mechanism which is driven by the large negative-inside membrane potential (–180 mV) maintained by mitochondrial respiration. The striking feature of the Ca^{2+} uniporter is its extremely high transport rate, perhaps limited only by mitochondrial respiration (Hutson et al., 1976; Crompton et al., 1977; Vercesi et al., 1978; McMillin-Wood et al., 1980; Gunter & Pfeiffer, 1990).

1.3.2.3.3 The mitochondrial Na^+/Ca^{2+} antiporter. The important role of calcium in cardiac muscle contraction/relaxation was well defined in the 1970s. It

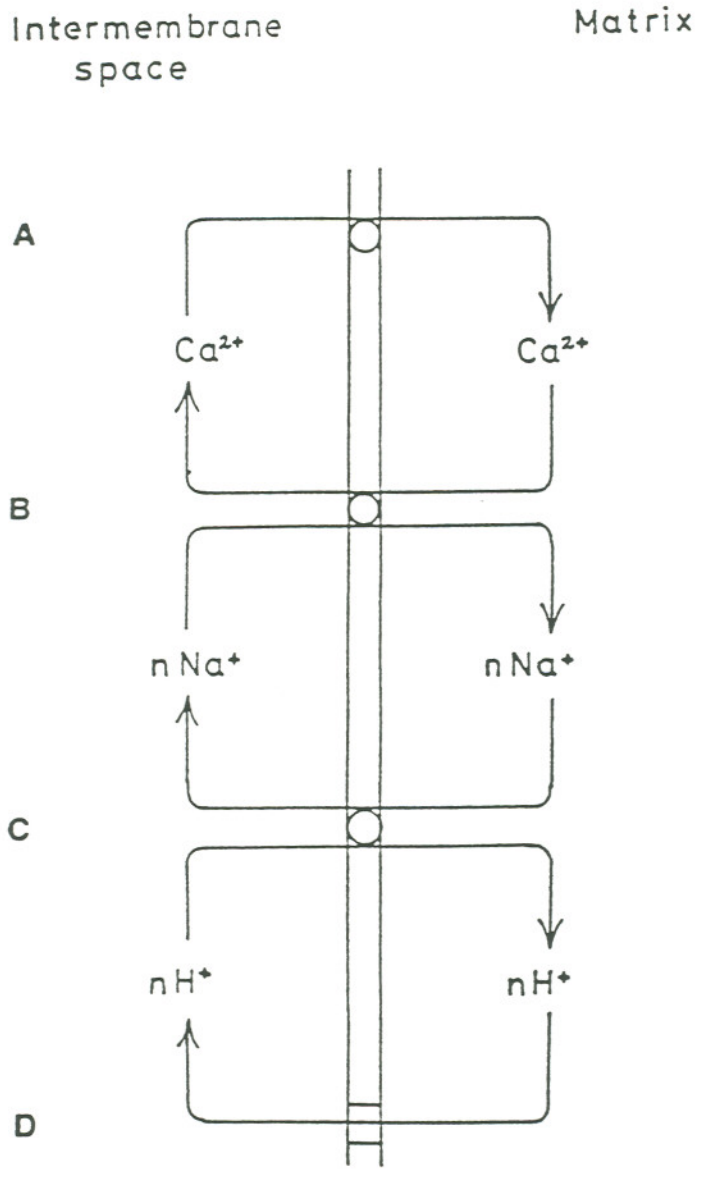

Figure 1.4 The heart mitochondrial Ca^{2+} transport cycle. **(A)** Ca^{2+} uniporter; **(B)** Na^+/Ca^{2+} antiporter; **(C)** Na^+/H^+ antiporter; **(D)** respiratory chain (Crompton, 1990).

was speculated by Carafoli and co-workers (1974) that mitochondria were a major source of an observed calcium release evoked by an extracellular sodium stimulus.

Crompton et al. (1977) re-examined the sodium-induced calcium efflux via Ca^{2+}-selective electrodes and reported the K_m for Na^+ was 8 mM with a maximum velocity of 15 nmol/mg/min. They were also able to detect the calcium-dependent sodium uptake into cardiac mitochondria and were first to suggest the existence of the Na^+/Ca^{2+} antiporter in cardiac mitochondria. Using sensitive fluorescent probe assays, Garlid's laboratory (Jezek et al., 1990; Garlid et al., 1991; Li & Garlid, 1991) was able to locate mitochondrial Na^+/Ca^{2+} exchange activity in distinct reconstituted fractions of solubilized mitochondrial proteins. They were further able to associate this Na^+/Ca^{2+} antiporter activity with a 110-kDa inner mitochondrial membrane protein.

The studies of mitochondrial calcium transport were originally performed using ion-selective electrodes, radioactive ^{45}Ca, and characteristic absorption. More recently, fluorescent probes such as indo-1 and fura-2 have been loaded into intact mitochondria (Davis et al., 1987; Lukacs & Kapus, 1987; Moreno-Sanchez & Hansford, 1988; Cox & Matlib, 1993). However, the complexity of mitochondria has made it difficult to assign an observed phenomenon as being due to the activity of a single protein, especially when the protein is not well characterized. The isolation and reconstitution of the Na^+/Ca^{2+} antiporter protein offered enormous advantage in this respect. Based on investigations in our lab and others [see details in Li (1992)], we have concluded that the mitochondrial Na^+/Ca^{2+} antiporter has a high turnover rate, consistent with its postulated role in the regulation and maintenance of steady-state matrix Ca^{2+} activity.

1.3.2.3.4 Regulation of the mitochondrial Na^+/Ca^{2+} antiporter. For the Na^+/Ca^{2+} antiporter to effectively regulate matrix Ca^{2+} activity, it must itself be tightly regulated. The sensitivity of the Na^+/Ca^{2+} antiporter to a variety of substances is consistent with this postulated function. The mitochondrial Na^+/Ca^{2+} antiporter is itself tightly regulated *in vivo*, and hormones, pharmacological agents, pH, and physiological ions are expected to be important in its regulation. In turn, the

well-regulated Na^+/Ca^{2+} antiporter manifests its significance in regulating mitochondrial Ca^{2+} cycling and metabolism.

The activation of the Na^+/Ca^{2+} antiporter by β-adrenergic agonists and glucagon in isolated mitochondria was reported (Goldstone & Crompton, 1982; Goldstone et al., 1983), but it was also found that the matrix Ca^{2+} level was not disturbed by these agents even though the Na^+/Ca^{2+} exchange was increased two- to three-fold. This indicated that the major action of glucagon and β-adrenergic agonists is to modulate the responsiveness of mitochondria to external Ca^{2+}. The result is the rapid cycling of Ca^{2+} across the inner membrane, enabling the mitochondria to respond more promptly to the cytosolic Ca^{2+} surges brought about by extracellular signals.

Under many experimental conditions, pH has a dramatic effect on enzymatic activity. The Na^+/Ca^{2+} antiporter has been demonstrated to be more active at lower pH (Rizzuto et al., 1987). Baysal et al. (1991), using the fluorescent probe BCECF to monitor matrix pH changes (together with nigericin to equilibrate pH across the membrane), found that Na^+/Ca^{2+} exchange exhibited maximum activity at pH 7.4–7.6. Our laboratory showed similar results in studies on the purified carrier, with highest Ca^{2+} uptake activity at pH 7.3. Both low and high pH dramatically reduce Na^+/Ca^{2+} exchange activity, primarily by increasing the K_m for calcium (W. Li et al., 1992). These data establish the significance of pH in regulating Na^+/Ca^{2+} exchange activity.

Hayat and Crompton (1982) found the maximal exchange rate for this process was decreased 70% by cytoplasmic calcium and suggested that the Na^+/Ca^{2+} antiporter might contain regulatory sites sensitive to extra-mitochondrial calcium in the physiological range of 10^{-4} mM.

The inhibitory effect of Mg^{2+} on the Na^+/Ca^{2+} antiporter is very profound and of physiological relevance. Hayat and Crompton (1987) found that, at cytosolic Mg^{2+} levels, the Na^+/Ca^{2+} antiporter could be effectively blocked and that this inhibitory effect could be abolished by ATP or ADP. This finding may provide a physiological bridge between the phosphorylation potential and calcium regulation of

mitochondrial ATP production. High matrix Ca^{2+} may activate the ATP production process and the phosphorylation potential may be increased. The increased ATP could then release the Na^+/Ca^{2+} antiporter from Mg^{2+} inhibition, decrease matrix Ca^{2+}, and result in the decline of ATP production.

Lukacs and Fonyo (1986) studied the effects of Ba^{2+} on both Na^+-independent Ca^{2+} efflux in liver mitochondria and Na^+-dependent Ca^{2+} efflux in heart mitochondria; they found that Ba^{2+} could inhibit both processes effectively. The benzothiazepines, particularly diltiazem, can effectively inhibit the Na^+/Ca^{2+} antiporter with similar potency in both intact mitochondria (Vaghy et al., 1982; Matlib & Schwartz, 1983; Matlib et al., 1983) and in the purified carrier (W. Li et al., 1992). Although these drugs are better known for their Ca^{2+} channel blocking activity, their effect on the mitochondrial Na^+/Ca^{2+} antiporter may also explain some of the important effects of these pharmacological agents.

1.3.2.3.5 Physiological significance of the Na^+/Ca^{2+} antiporter. The hypothesis that mitochondrial ATP production is primarily regulated by matrix calcium activity dictates the vital role played by the calcium cycle (Denton et al., 1972, 1978; McCormack & Denton, 1979). It is expected that cellular energetic states can be shifted by altering the steady state of the calcium cycle. Among the four components of this cycle mentioned above, the Na^+/Ca^{2+} antiporter and Ca^{2+} uniporter are directly linked to calcium transport and are thus likely to be significant in Ca^{2+} cycle regulation. The expected physiological levels of Ca^{2+} in the cytosol and mitochondrial matrix indicate that the Ca^{2+} uniporter operates at conditions far from equilibrium. The extremely high turnover rate of the Ca^{2+} uniporter makes it an excellent candidate to enable mitochondria to rapidly respond to cytosolic Ca^{2+} activity changes. On the other hand, it is known that mitochondrial Ca^{2+} does not follow the cytosolic Ca^{2+} surges instantly; hence the Na^+/Ca^{2+} antiporter must play a central role in regulating the matrix Ca^{2+} activity.

This point was elucidated by Cox and Matlib (1993) in their study of the Na^+/Ca^{2+} antiporter in isolated mitochondria. Matrix Ca^{2+} activity was monitored by fura-2 loaded into mitochondria. External sodium was varied to induced Ca^{2+}

efflux via the Na^+/Ca^{2+} antiporter, thus demonstrating this protein to be responsible for matrix Ca^{2+} activity changes. In addition, the corresponding tricarboxylate cycle turnover rate (supported by α-ketoglutarate dehydrogenase) and the oxidative phosphorylation rate were also found to change according to Na^+/Ca^{2+} exchange activity. With the regulation of the tricarboxylate cycle and oxidative phosphorylation by Ca^{2+} established, the mitochondrial Ca^{2+} transport system was assigned the role of regulating matrix Ca^{2+} activity (Denton & McCormack, 1985; Hansford, 1985). This study has confirmed the hypothesis and clearly indicates that the Na^+/Ca^{2+} antiporter is a major regulatory site of the mitochondrial Ca^{2+} cycle.

The proposed role of the Na^+/Ca^{2+} antiporter in the regulation of matrix Ca^{2+} activity requires that it, in turn, be tightly regulated. This appears to be the case, as discussed earlier. This regulatory mechanism offers the possibility of modulating mitochondrial ATP production. Since ATP is the energy form available to the cell, this would allow modulation of the rate of cellular processes.

1.3.2.3.6 The significance of the Na^+/Ca^{2+} antiporter in pathological situations. The physiological importance of Ca^{2+} in mitochondria and the mechanism of Ca^{2+} transport systems, especially the Na^+/Ca^{2+} antiporter, warrants some discussion of their role in pathological conditions.

Mitochondria isolated from the ischemic myocardium have a defective capacity for oxidative phosphorylation, and further impairment occurs upon reperfusion. Such mitochondria also contain increased Ca^{2+} (Henry et al., 1977). Some experiments have shown that reperfusion with ruthenium red largely prevents both increase in mitochondrial Ca^{2+} and impairment of oxidative phosphorylation, and improves myocardial contractility (Peng et al., 1980; Smith, 1980; Ferrari et al., 1982). Taken as a whole, these studies suggest that ischemia/reperfusion is associated with increased mitochondrial Ca^{2+} and that prevention of this increase might be beneficial.

The consequences of mitochondrial Ca^{2+} overload are well known: mitochondria become uncoupled, membrane potential is dissipated, and oxidative phosphorylation is inhibited. Because the maximum rate of Ca^{2+} cycling, which is fixed by the maximum rate of Na^+/Ca^{2+} exchange, would give rise to no more than a

2% increase in respiratory activity, the Ca^{2+}-induced uncoupling of respiration and dissipation of membrane potential must be caused by some effect of Ca^{2+} *per se* rather than by Ca^{2+} flux. In fact, it has long been known that, with accumulation of excess Ca^{2+}, isolated mitochondria swell and generally become leaky to low-molecular-weight solutes, such as K^+, Mg^{2+}, and adenine and pyrimidine nucleotides. This nonspecific leakiness undoubtedly accounts for the uncoupling of energy transduction.

Two hypotheses have been presented for the mechanism of this permeabilization. In the phospholipase model of permeabilization (Broekemeier et al., 1985), mitochondria contain Ca^{2+}-dependent phospholipase A_2, some of which appear to be located on the inner face of the inner membrane. Perhaps the most obvious way in which Ca^{2+} might induce nonspecific leakiness is via limited degradation of the inner membrane bilayer. Some groups (Riley & Pfeiffer, 1986) have proposed cyclic deacylation/reacylation of membrane phospholipids catalyzed by phospholipase A_2 (deacylation) and by acyl-CoA synthetase plus lysophospholipid acyltransferase (reacylation). Accordingly, Ca^{2+}-induced displacement of the cycle towards deacylation is the root cause of permeabilization.

In the pore model of permeabilization, it is hypothesized that mitochondria might contain a nonspecific "channel" regulated by Ca^{2+}, as was first suggested by Haworth and Hunter (1980). More recently, Al Nasser and Crompton (1986) examined the critical question of the reversibility of permeabilization and suggested that permeabilization/resealing reflected the presence of a reversible Ca^{2+} activated pore. Their further studies confirmed that Ca^{2+} removal alone sufficed for rapid closure of the hypothetical pore, finding that the rate of closure was markedly stimulated by ADP (Crompton & Costi, 1988).

In all probability, however, pore opening is a more complex process. Promotion of inner membrane permeabilization by redox-coupled oxidation has already been noted. Direct assays of pore states have shown that, although Ca^{2+} is required, it alone is quite unable to induce pore opening and that either inorganic phosphate or oxidative stress is also required (Crompton & Costi, 1988). Evidently

the pore is not simply activated by Ca^{2+}. It has been suggested that high Ca^{2+} concentrations may induce a loss of substrate selectivity by the adenine nucleotide translocase transport system (Le Quoc & Le Quoc, 1988).

It is generally agreed that Ca^{2+} overload, oxidative stress, and increased tissue inorganic phosphate are major factors in reperfusion injury. As mentioned earlier, cytosolic free Ca^{2+} concentration rises after prolonged anoxia (Allshire et al., 1987). In whole tissue, reperfusion induces massive Ca^{2+} uptake, whereas other small molecules are not taken up (Poole-Wilson et al., 1984), suggesting a defective capacity of the plasma membrane for active Ca^{2+} transport (see Figure 1.5).

It has been suggested that, owing to a decreased cytosolic phosphorylation potential, impaired active Ca^{2+} transport by the plasma membrane and SR is a major factor in the inability of heart cells to maintain Ca^{2+} homeostasis after prolonged ischemia/reperfusion (Crompton, 1990). If this is the case, then mitochondrial pore opening would surely be catastrophic, as any pore opening would lead indirectly to increased net Ca^{2+} influx into the cell, which in turn would promote additional pore opening. The situation would amount to a vicious downward spiral of phosphorylation potential and capacity for Ca^{2+} control.

1.4 Biochemical and Molecular Biological Studies of the Na^+/Ca^{2+} Antiporter

The identification and purification of the Na^+/Ca^{2+} antiporter marked a major advance in the study of membrane transport proteins and opened doors for further characterization (Li & Garlid, 1991). Advances in molecular biology have provided new means by which to study membrane ion transporters. An excellent example is the Na^+/Ca^{2+} exchanger in the plasma membrane. Philipson's group successfully purified the cardiac sarcolemmal Na^+/Ca^{2+} exchange protein (Philipson et al., 1988). Purification studies on heart sarcolemma (Hale et al., 1984; Soldati et al., 1985) and brain synaptosomes (Barzilai et al., 1984) developed a variety of techniques for purification, identification, and reconstitution of the Na^+/Ca^{2+} exchange protein. Nicoll et al. (1990) reported the molecular cloning, deduced amino acid sequence, and

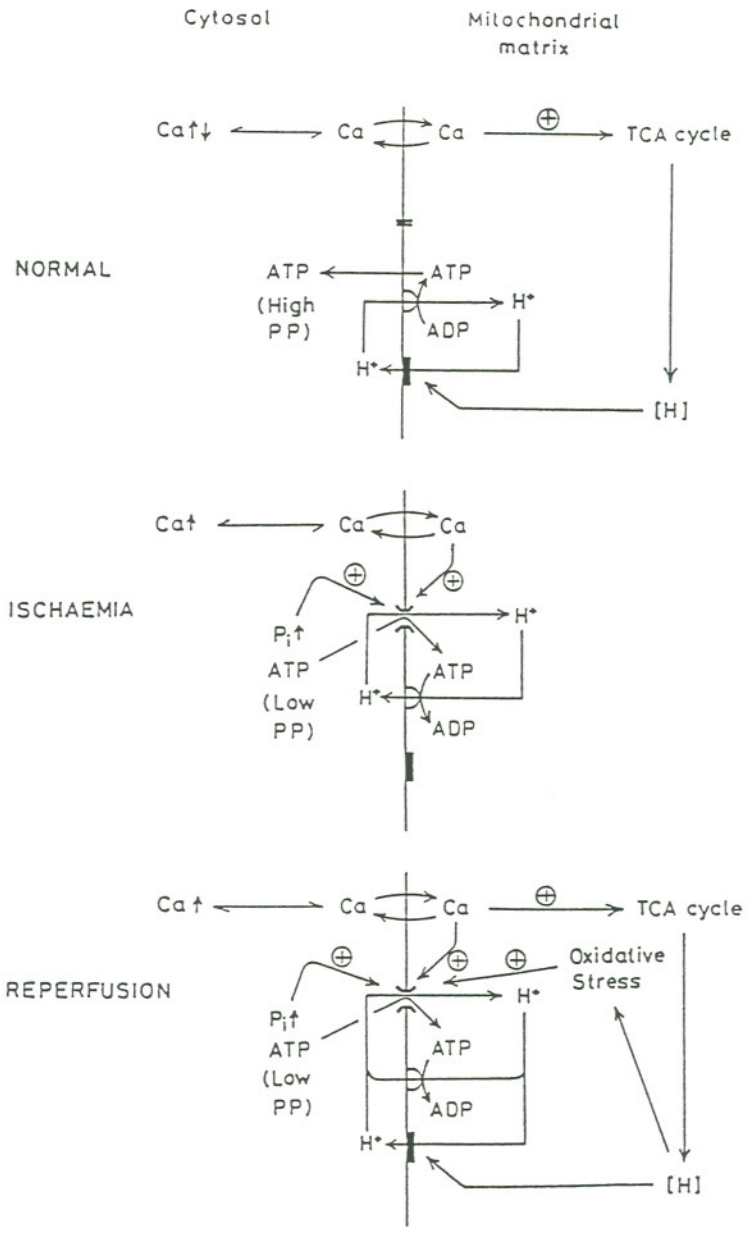

Figure 1.5 An hypothesis for disruption of mitochondrial energy transduction during ischemia and reperfusion. Abbreviations: ↑, increase; ↓, decrease; ⊕, activation; [H], reducing equivalents; PP, phosphorylation potential. Under normal conditions, the inner membrane pore is closed (=), and H^+ cycling via the respiratory chain (≈) and the F_1ATPase (D) generates a high cytoplasmic PP. In ischemia and reperfusion, pore opening (=) short-circuits H^+ cycling and allows hydrolysis of cytoplasmic ATP. The resultant decrease in PP impairs active Ca^{2+} transport by the plasma membrane and sarcoplasmic reticulum (not shown), cytoplasmic Ca^{2+} rises further, and pore opening is further promoted (Crompton, 1990).

apparent molecular size of the canine cardiac sarcolemmal Na^+/Ca^{2+} antiporter. The open reading frame of 2910 base pairs encodes a protein of 970 amino acids with a molecular size of 108 kDa. cDNA cloning and functional expression have resulted in major progress in the characterization of the sarcoplasmic Na^+/Ca^{2+} antiporter. After cloning of the Na^+/Ca^{2+} antiporter from various tissues (including human myocardium) was accomplished (Aceto et al., 1992; Kofuji et al., 1992; Reilander et al., 1992), the gene of the cardiac Na^+/Ca^{2+} antiporter was expressed in mammalian cells (Kofuji et al., 1992, 1993). The molecular biology, cellular function, and the role of this carrier have been studied by Z. Li et al. (1992) and Schulze et al. (1993). A gene structure–function model for this carrier has been analyzed by Kofuji's group (Kofuji et al., 1994).

1.5 Review of Mitochondrial Na^+/Ca^{2+} Antiporter Studies in Our Lab

Despite increasing recognition of the importance of the mitochondrial Na^+/Ca^{2+} antiporter in the regulation of matrix Ca^{2+} (which in turn controls the processes needed to respond to cellular demands for ATP and to maintain normal membrane permeability to the small molecules), very little is as yet known about the molecular nature of this carrier. Many groups (i.e., McCormack and Matlib) including our own (Garlid) are working to purify and express the mitochondrial Na^+/Ca^{2+} antiporter. Our lab first reported partial purification of a reconstituted active Na^+/Ca^{2+} antiporter protein as a 110-kDa protein from beef heart mitochondria (W. Li et al., 1992).

While studying the Na^+/H^+ antiporter (Garlid et al., 1991), we first observed Na^+/Ca^{2+} exchange in proteoliposomes reconstituted with whole extract from beef heart submitochondrial particles. We successfully partially purified the protein with this activity by reconstituting chromatographic fractions from DEAE-cellulose columns. Na^+/Ca^{2+} exchange activity was studied using the Na^+-selective fluorescent probe SBFI and the Ca^{2+}-selective fluorescent probe fura-2. Reconstituted Na^+/Ca^{2+} exchange was found to be inhibited by diltiazem, and TPP^+ (as observed

in intact mitochondria) was found to be able to catalyze Na^+/Li^+ exchange in the absence of Ca^{2+}. Polyclonal antibodies were raised against a 110-kDa protein associated with this activity, and these were shown to inhibit the Na^+/Ca^{2+} exchange (W. Li et al., 1992).

Screening of a λgt11 cDNA expression library of beef heart, using the 110-kDa protein specific polyclonal antibodies as a probe, resulted in positive clones which later proved to be mitochondrial nicotinamide nucleotide transhydrogenase (TH) (Cheng, 1995). This led to the realization that the 110-kDa protein band containing the putative Na^+/Ca^{2+} antiporter also contained TH (in considerable abundance), and we concluded that the 110-kDa band specific antibodies were likewise a mixture of TH and Na^+/Ca^{2+} protein antibodies. To separate the two proteins in the 110-kDa band, a partial TH antigen was expressed in *E. coli* and antibodies against it were immuno-purified. Two 110-kDa proteins of differing isoelectric points were distinguished by Cheng (1995).

To remove TH contamination from the 110-kDa protein mixture, a TH antibody immobilized chromatography column was used to absorb the TH protein from the 110-kDa protein fraction mixture. The resultant flow-through fraction contained mainly the putative Na^+/Ca^{2+} antiporter with little TH contamination. The antibody specific to this "negatively" purified 110-kDa protein showed inhibition of Na^+/Ca^{2+} transport, while the TH antibody did not (data not shown). This was the status of the project at the beginning of the work reported in this thesis.

My work, based on our previous work on the Na^+/Ca^{2+} antiporter, has been to further refine the purification procedures and thereby permit us to obtain the internal peptide sequence of the 110-kDa Na^+/Ca^{2+} mitochondrial transport protein.

HA chromatography, with SDS as the detergent in the phosphate buffer, was introduced to "purify" the 110-kDa protein in the wash fractions from the second DEAE-cellulose column. HA has the advantage of concentrating the protein without the detergent being concentrated and losing protein. Until now, procedures to purify the 110-kDa protein led to fractions with elevated detergent levels (over 12% Triton X-100). These high detergent levels impaired the immuno-affinity column. As the

HA column cannot separate 110-kDa protein from proteins with other molecular weights, electrophoretic separation was used to isolate them from the 110-kDa band prior to immuno-purification. Additionally, other technical refinements and improvements were made and are discussed in the sections that follow.

The existence of a Na^+/Ca^{2+} antiporter was established more than 25 years ago. Since then, a considerable amount of information has been obtained regarding the exchanger's mode of action, its role in cellular Ca^{2+} homeostasis, regulation of ATP production, and distribution, not only in excitable and secretory cells but also in a large number of other cell types (Rahamimoff, 1989). Elucidation of the molecular biochemistry of this Na^+/Ca^{2+} antiporter, however, has a much shorter history. In particular, two major difficulties have retarded progress in such investigations.

In terms of abundance, the Na^+/Ca^{2+} antiporter is a minor membrane component. Estimates of site density in cardiac sarcolemma and determination of the amount of purified protein obtained from synaptic membranes indicate that the Na^+/Ca^{2+} antiporter is less than 0.1% of total membrane proteins (Barzilai et al., 1984, 1987). In the mitochondrial membrane its abundance appears to be even less. In the absence of a rich source for this protein, an inordinately large quantity of membrane must be processed in order to obtain sufficient amounts of the purified protein for biochemical procedures.

A second, even more serious difficulty is the lack of a specific inhibitor, toxin, or affinity label that can be used to identify the purified protein through specific binding. Thus while several pharmacological agents have been found to inhibit the Na^+/Ca^{2+} antiporter (amiloride and its derivatives, doxorubicin and verapamil among them), none is specific or usable as an exclusive probe to label the antiporter protein. So far, demonstration of Na^+/Ca^{2+} antiporter activity is the sole index for detection of its presence. As a consequence, purification of the Na^+/Ca^{2+} antiporter protein requires solubilization of the appropriate plasma membrane or other membrane separating the compartment of the organelle (in our case the mitochondrial inner membrane), followed by functional reconstitution of the isolated putative proteins into the phospholipid membrane to demonstrate transport activity, and identification of the

specific protein entity responsible for that activity. In view of these difficulties, development of native and reconstituted membrane vesicle preparations has preceded attempts at purification of the Na^+/Ca^{2+} antiporter.

CHAPTER 2
EXPERIMENTAL MATERIALS AND METHODS

2.1 Materials

DEAE-cellulose, SDS and ultra pure SDS, Triton X-100 and reduced Triton X-100, alkaline phosphatase conjugated antibody, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Sigma. HA chromatographic powder and 10-ml disposable columns, SDS-PAGE electrophoresis reagents and apparatus, western blot transfer apparatus and reagents, and a Model 422 electrophoretic-eluter apparatus were purchased from Bio-Rad. Leupeptin, anti-rabbit Ig, horseradish peroxidase-linked whole antibody, and ECL western blot reagents were from Amersham Inc., PMSF from Boehringer Mannheim Biochemical, and dialysis membranes from Spectro/Por. An Immuno-Pure Ag/Ab immobilization kit, microdialyzer system and membrane were obtained from Pierce. A stirred-cell ultrafiltration apparatus, membrane and Centricon concentrators were purchased from Amicon. Other routine chemicals, buffers and reagents were supplied by Sigma or Fisher.

2.2 Purification of the Beef Heart Mitochondrial Na^+/Ca^{2+} Antiporter

2.2.1 Isolation of beef heart mitochondria and submitochondrial particles

Starting material for the Na^+/Ca^{2+} protein purification consisted of submitochondrial particles (SMP) from fresh bovine heart mitochondria isolated following the procedures of Azzone et al. (1979) as modified by Garlid et al. (1991). All these and subsequent purification procedures were carried out at 4°C unless otherwise noted.

2.2.2 Purification by DEAE-cellulose chromatography

2.2.2.1 ***Solubilization of SMP.*** SMP membrane protein (200 mg) was solubilized and extracted for 60 min by incubation with stirring (4 mg protein/ml) in a buffered detergent medium consisting of 50 mM TEA-TES (pH 7.3), 1 mM TEA-EDTA, 0.1 mM DTT, 20% (v/v) glycerol, 3% (v/v) Triton X-100, supplemented with pepstain A and leupeptin (1 μg/ml and 10 μg/ml, respectively) to protect against protease degradation. The solubilized SMP extract was centrifuged for 30 min at 130,000 $\times$ g to remove membrane fragments, and the supernatant was collected for chromatographic purification.

2.2.2.2 ***Preparation of the DEAE-cellulose column.*** DEAE-Cellulose, pretreated according to the supplier's recommendations (Sigma) was pre-equilibrated with column buffer: 50 mM TEA-TES (pH 7.3), 1 mM TEA-EDTA, 0.1 mM DTT, 1% (v/v) Triton X-100 detergent, and 20% (v/v) glycerol. Fines were removed, and the column was poured and allowed to settle under gravity until a 20-ml bed volume was established. The column was then washed with column buffer until the pH of the outlet solution stabilized at 7.3 prior to loading of the SMP extract.

2.2.2.3 ***Separation of the 110-kDa protein by DEAE-cellulose chromatography.*** Approximately 200 mg total protein SMP extract was loaded onto the column at a rate of 0.5 ml/min. The flow-through was discarded and wash fractions were collected as the loaded column was washed with an additional 3 bed (column) volumes (60 ml) of column buffer. The wash fractions, shown previously to contain the 110-kDa Na^+/Ca^{2+} protein activity (W. Li et al., 1992), were then pooled, divided into 4 equal volumes ($\sim$ 15 ml each), and each part then subjected to a second chromatography on a new separate DEAE-cellulose 10-ml column using the same procedures as above. Wash fractions from the second columns were again pooled and concentrated 3:1 by stirred-cell ultrafiltration using a 10-kDa cut-off membrane and 170 kPa nitrogen. Aliquots of the concentrate were taken for protein concentration measurement [typically 100 μg/ml by the amido black method (Kaplan & Pedersen, 1985)] as well as for SDS-PAGE verification of the presence of the 110-kDa protein (Figure 3.1 A). Protease inhibitors such as pepstain A, leupeptin, and

PMSF (1 μg/ml, 1 μg/ml, and 100 μg/ml, respectively) were added as protective agents to the concentrated protein sample. Ultra-pure SDS powder (0.2%) was added in this sample to 0.2% (w/v) just before loading the protein sample onto an HA column.

2.2.3 Concentration of 110-kDa protein by HA chromatography

2.2.3.1 ***HA column preparation.*** HA was first equilibrated with a low ionic strength buffer of 10 mM sodium phosphate, 5 mM DTT, and 0.2% (w/v) ultra-pure SDS (pH 6.4). After decanting fines, the column was poured, allowed to settle to a bed volume of 3 ml ($\sim$1 g dry HA), and extensively washed with 10 bed volumes of the buffer.

2.2.3.2 ***Chromatographic procedures.*** The collected concentrated wash fractions from the second DEAE-cellulose columns (**2.2.2.3**, above) were loaded onto the prepared HA column ($\sim$1 mg total protein/ml of dry HA) and extensively washed with 6 bed volumes of the column buffer to remove the Triton X-100. A step gradient, 2 bed volumes per step, with increasing ionic strengths of 100, 150, 200, 250, 300, 400, 500 mM sodium phosphate buffer (pH, DTT and SDS held constant), was used to elute the protein into fractions of ½ bed volume each (4 fractions per step). SDS-PAGE analysis of the collected fractions (Figure 3.2 A) showed that the 110-kDa protein was eluted in the 400 mM sodium phosphate fractions. After pooling the relevant fractions, protein concentration was measured at $\sim$150 μg/ml. The fractions containing the 110-kDa protein were then denatured by the addition of 1 volume of sample buffer [3.3% ultra-pure SDS, 166.6 mM Tris-HCl (pH 6.7), 30% (v/v) glycerol, 5% (v/v) β-mercaptolethanol] to each 2 volumes of the fraction, heating at 90°C for 3 min, and finally stored at -70°C.

2.2.4 SDS-PAGE and electrophoresis-elution isolation of the 110-kDa band protein from the column-purified sample

2.2.4.1 ***Gel purification procedures.*** Polyacrylamide vertical slab gels (7.5%) were prepared according to the procedures of Laemmelli (1970). The sample

containing the 110-kDa partially purified protein was loaded in single lanes (400–600 μg per gel) and run with a standard buffer solution (25 mM Tris, 200 mM glycine, 0.2% (w/v) ultra-pure SDS) for 1.5 h. The protein bands were visualized with 0.2% (w/v) Coomasie Brilliant-Blue (in 40% methanol, 10% acetic acid) for 1 h, destained for 1–2 h with two or three changes of destaining solution, and washed with deionized water. The 110-kDa band was then physically excised and cut into small pieces for electro-elution.

2.2.4.2 ***Elution of the 110-kDa protein from the gel slices.*** The excised SDS-PAGE 110-kDa band gel pieces were electro-eluted from the gel pieces using a Bio-Rad Model 422 Electrophoretic Eluter for 8–10 h at 8–10 mA/cell in an ice cold eluting solution (25 mM Tris, 200 mM glycine, 0.2% (w/v) ultra-pure SDS). The eluted protein was carefully collected, dialyzed to effect a change of detergent, and concentrated in preparation for immuno-affinity purification (see details, below in **2.2.6**).

2.2.5 Extraction of specific antibodies from 110-kDa protein antiserum

2.2.5.1 ***Extraction of the TH-specific antibody from the 110-kDa protein antiserum.*** Starting material for preparation of the TH-specific antibody consisted of pure TH protein, which was previously expressed via *E. coli* (Cheng, 1995), and antiserum previously raised against the 110-kDa protein mixture (W. Li et al., 1992). The actual method used was a modification of traditional band-purification techniques [see Hager & Burgess (1980) and Smith & Fisher (1984)], wherein the pure TH protein was used to "select out" TH-specific antibody protein from the 110-kDa protein antiserum.

2.2.5.1.1 Bacterial expression of the recombinant protein. A single transformant colony, confirmed by enzyme mapping to verify that the recombinant pQE plasmid did contain the insert, was inoculated into LB medium containing 25 μg/ml kanamycin and ampicillin and grown at 37°C overnight. A short second growth was inoculated 1:50 with the overnight culture and was grown at 37°C with vigorous shaking until the A_{600} reached 0.7–0.9. IPTG was added to a final

concentration of 1–2 mM to induce expression of the recombinant protein, and incubation was continued for a further 4 h. A control culture containing the pQE-16 transformant, which expresses the 26-kDa dihydrofolate reductase (DHFR), was grown at the same time. The cells were harvested by centrifugation; an aliquot for SDS-PAGE analysis was removed and then frozen until purification.

2.2.5.1.2 Purification of recombinant protein. The expressed proteins containing six consecutive histidine residues (the 6 His affinity tag) at their N-terminus were purified by Ni-NTA resin with high affinity ($K_d = 10^{-3}$, pH 8.0).

Cells were resuspended in a guanidine buffer (6 M guanidine-HCl, 0.1 M $NaPO_4$, and 0.01 M Tris/HCl, pH 8.0) at 5 ml/g wet weight, then stirred for 1 h at room temperature. The cell lysate was centrifuged at 10,000 × *g* for 15 min at 4°C, then Ni-NTA resin was added to the supernatant (8 mg of 50% slurry of Ni-NTA resin for a 500-ml culture), stirred at room temperature for 45 min, and loaded into a 1.6-cm-diameter column. The column was washed with 10 column volumes of guanidine buffer, 5 column volumes of urea buffer (8 M urea, 0.1 M NaPO, 0.01 M Tris/HCl, pH 8.0) and urea buffer (pH 6.3) until the A_{280} was less than 0.01. The recombinant protein, which possess 6 His residues at its N-terminal and thus can be bound by Ti-NTA resin, was then eluted with 10–20 ml of urea buffer (pH 5.9), followed by 10–20 ml urea buffer (pH 4.5), and 20 ml of final buffer (6 M guanidine-HCl, 0.2 M acetic acid). (TH expressed protein was mainly contained in the urea buffers at pH 5.9 and 4.5.) From each elution, 3-ml fractions were collected and analyzed by 10% SDS-PAGE and by western blot with the 110-kDa TH antibody. Additional details can be found in the thesis of Cheng (1995).

Pure TH protein obtained by *E. coli* expression was denatured and run on a 10% SDS-PAGE preparative gel. The protein was then transferred onto a methanol-pretreated PVDF membrane for 1 h at 230 mA using a transfer buffer (20% methanol, 50 mM Tris, 400 mM glycine). After transfer, the membrane was briefly exposed (1 min) to Coomasie Brilliant-Blue staining solution, then thoroughly destained, and finally rinsed in deionized water. This procedure resulted in a clearly

delineated 39-kDa band, identifiable as the partially expressed TH protein (see Cheng, 1995).

The portion of the membrane containing the expressed TH protein was carefully excised and cut into small pieces which were then blocked in 5% non-fat milk in TBS for 1 h at room temperature and washed three times in TBST. The antiserum containing polyclonal antibodies raised against the 110-kDa proteins (W. Li, 1992) was then added to the blocked membrane pieces and gently stirred overnight at 4°C. After incubation, the antiserum was decanted and the membrane pieces, which contained the TH protein/TH-specific antibody conjugate, were washed five times with 1% non-fat milk in TBST (10 min/wash). After a quick final rinse in TBS, the TH-specific antibody was removed by two successive elutions using a solution of 100 mM NaCl and 100 mM glycine (pH 2.5). The eluate was immediately "neutralized" to pH 7.5 with 1 M Tris/HCl (pH 8.8) and checked for protein concentration by UV-absorbance at 280 nm (Harlow & Lane, 1988). NaN_3, 0.05% final concentration, was added and the eluate was stored at −20°C. The membrane pieces containing the TH protein were re-equilibrated in TBST to pH 7.5, washed several times, and re-incubated with the antiserum, as above. This cycle was repeated (up to ten times per membrane) until sufficient TH-specific antibody had been collected.

Repeated extraction of the TH-specific antibody from the antiserum yielded ~5–7 mg of total antibody protein in a volume of ~70 ml. This volume was reduced to a more manageable 10 ml by stirred cell ultrafiltration with a membrane filter cut-off of 50 kDa at 4°C under nitrogen. The concentrate was dialyzed overnight at 4°C against 100 mM sodium phosphate (pH 7.4). The buffer was changed three times to remove Tris (which interferes with the immuno-affinity column's conjugation of the antibody protein). The final TH-specific antibody volume was reduced to 2 ml using a Centricon concentrator (50-kDa cut-off). During this final step, a minor precipitate was noted, and the sample was centrifuged (15,800 × *g* at 4°C for 20 min) to remove it. NaN_3 (0.05%) was again added, and the TH-specific antibody was frozen at −70°C.

2.2.5.2 ***Extraction of the enriched Na^+/Ca^{2+}specific antibody from the 110-kDa protein antiserum.*** Using the above procedures, the antibody against the purified Na^+/Ca^{2+} antiporter protein was obtained by using "enriched" Na^+/Ca^{2+} antigen protein (Cheng, 1995) in lieu of that against the expressed TH protein. This enriched Na^+/Ca^{2+} antibody was later used in western blot analysis to verify the separation of the Na^+/Ca^{2+} antiporter from the TH protein by the immuno-affinity column procedures.

2.2.6 Purification of the Na^+/Ca^{2+} antiporter by immuno-affinity chromatography

2.2.6.1 ***Sample preparation for the affinity column: detergent replacement.*** The sample containing the 110-kDa protein mixture was dialyzed in a Pierce microdialyser system against a PBS buffer with 0.2% reduced Triton X-100 and 0.05% NaN_3 for three days at room temperature (four buffer changes) to substitute Triton detergent for SDS. Following dialysis, the sample volume was reduced to 1.5 ml using a 50-kDa cut-off Centricon concentrator.

2.2.6.2 ***Preparation of the immuno-affinity column.*** Immuno-affinity purification of the Na^+/Ca^{2+} protein was effected using a Pierce Immuno-Pure Aminolink™ Antigen/Antibody Immobilization Kit, with some modification of the supplier's protocols. These procedures were carried out at room temperature.

Preparation of the affinity column required for separation of the Na^+/Ca^{2+} protein from the TH contaminant in the 110-kDa protein mixture is a two-step procedure. First, it was necessary to prepare an amount of TH-specific antibody sufficient to completely remove the TH protein contaminant from the 110-kDa protein mixture. Second, it was necessary to fix this TH-specific antibody to the aminolink immuno-pure column, which would then be used to perform the actual Na^+/Ca^{2+} purification.

The storage solution was drained from a 2-ml agarose gel Immuno-Pure column and the column then equilibrated with 6 ml of coupling buffer (0.1 M phosphate, pH 7.0). Then 2 ml of solution containing the purified TH antibody

(~5–7 mg antibody protein) was added to the column. The amount of TH antibody required was calculated from the total amount of 110-kDa protein to be subsequently loaded onto the column, multiplied by a factor of 25 to ensure excess TH binding capacity.

After addition of 0.2 ml of freshly prepared reducing reagent (1 M $NaCrBrH_3$, in 10 mM NaCl), the antibody/agarose slurry was incubated for 18 h with gentle end-to-end rocking. The effluent was collected, and the column was then washed with 2 ml of coupling buffer. Individual measurement of protein in the original TH antibody sample, in the post-incubation effluent, and in the wash fraction permitted calculation of the coupling efficiency of the column. This consistently ranged between 75 and 80% on the several occasions that this procedure was executed.

Next, any unoccupied column binding sites were blocked by the addition of 4 ml of quenching buffer (1 M Tris/HCl, pH 7.40) followed by 2 ml of quenching buffer together with 0.2 ml of reducing reagent. The column was gently mixed for 30 min, drained, and extensively washed with 20 ml of 1 M NaCl. Finally, 2 ml of eluting buffer (0.1 M glycine, 0.2% reduced Triton X-100, pH 2.8) was passed through the column, followed immediately by continued washing with PBS (also with 0.2% reduced Triton X-100, pH 7.4) until the pH of the effluent stabilized at 7.4. The TH-antibody-loaded agarose gel was stored at 4°C in PBS solution containing 0.5% NaN_3 until needed.

2.2.6.3 ***Purification of the Na^+/Ca^{2+} protein: affinity column removal of TH protein from the 110-kDa protein mixture.*** The TH antibody-loaded column was initially washed with 6 ml of PBS + 0.2% Triton, which was then removed by gentle centrifugation at 800 × *g* for 60 s. The 1.5 ml sample containing the 110-kDa protein mixture was added to the column, together with 0.5 ml of PBS + 0.2% Triton, and the slurry was gently mixed until uniformly suspended. After 4 h of gentle incubation at room temperature with horizontal rocking, the gel was allowed to settle with the column in a vertical position.

The column flow-through was collected and the column washed with 0.8 ml of PBS + 0.2% Triton buffer. The flow-through and wash volumes were combined and

held for a second pass through the column. The column was extensively washed with 16 ml of PBS + 0.2% Triton, and any residual TH protein was removed with 4 ml of elution buffer (0.1 M glycine, pH 2.8). This eluates were collected and stored for western blot analysis.

After eluting of TH protein from the column, the column was immediately washed with column buffer until the collected effluent stabilized at pH 7.4. The column was re-centrifuged as above, and the combined flow-through and wash volumes (~2.5 ml) from the first pass were re-loaded onto the column. The 110-kDa protein sample was subjected to a second purification on the "regenerated" affinity column. After incubation, the flow-through was obtained by slow centrifugation of the column (800 × *g* for 1 min), after which it was washed with 16 ml of column buffer. The collected purified protein sample fractions (now containing the Na^+/Ca^{2+} protein from which the TH protein had been removed) were stored at –70°C for western blot analysis. Any residual TH protein was again eluted from the column, and the column was re-equilibrated with column buffer to pH 7.4 and stored at 4°C with 0.05% NaN_3.

2.2.6.4 ***Western blot analysis of the affinity column products.*** The samples of flow-through and wash fractions from the second pass (containing Na^+/Ca^{2+}) and elution fractions (containing TH protein) from the first and second passes were subjected to western blot analysis with the two available antibodies: the TH-specific antibody and the "enriched" Na^+/Ca^{2+}-specific antibody (see **2.2.5** above), some parts of which were derived from Cheng (1995).

2.2.7 Sample dialysis and concentration

The sample containing the 110-kDa Na^+/Ca^{2+} antiporter protein from the second pass affinity-column flow-through was dialyzed for 4 h against PBS buffer with 0.2% reduced Triton-100 and 0.05% NaN_3 to remove salts and any protease inhibitors which might interfere with the digestion procedures associated with peptide sequencing. After dialysis, the sample volume was reduced to 0.5 ml (by a factor of 5) using a 50-kDa cut-off Centricon concentrator. The final sample contained ~100 μg of the Na^+/Ca^{2+} protein in 1% reduced Triton-100.

CHAPTER 3
RESULTS

3.1 Overview

Many laboratories, including our own, have been working on the biochemical and molecular biological characterization of various manifestations of the Na^+/Ca^{2+} antiporter for many years. My efforts, based on the previous work of W. Li et al. (1992) and Cheng (1995), has sought to improve upon their protein purification methods and to introduce new ones where appropriate. More specifically, my work has focused on improving the procedures and methods involved with the affinity column purification of the protein. These efforts proved to be very time-consuming, and, while some difficulties yet remain to be overcome before the sequencing of this antiporter can proceed, progress has been made.

3.2 Partial Purification of the Na^+/Ca^{2+} Antiporter at 110 kDa by DEAE-Cellulose Chromatography

The methods used for solubilization of mitochondrial membrane proteins and partial purification of the 110-kDa Na^+/Ca^{2+} antiporter were based on protocols previously developed in our laboratory, as described by W. Li et al. (1992). Initial trials (experiments) with the detergent concentrations in the membrane solubilization procedure indicated that significant improvement in 110-kDa protein recovery was possible. Thus, increasing the concentration of Triton-X 100 from 1% to 3% (v/v) in the SMP extraction, and thereby increasing the detergent/SMP protein ratio (w/w) from 10:4 to 10:1, resulted in a 40% increase in total protein extracted. Importantly, this modification did not change the established purification profile (see Figure 3.1A);

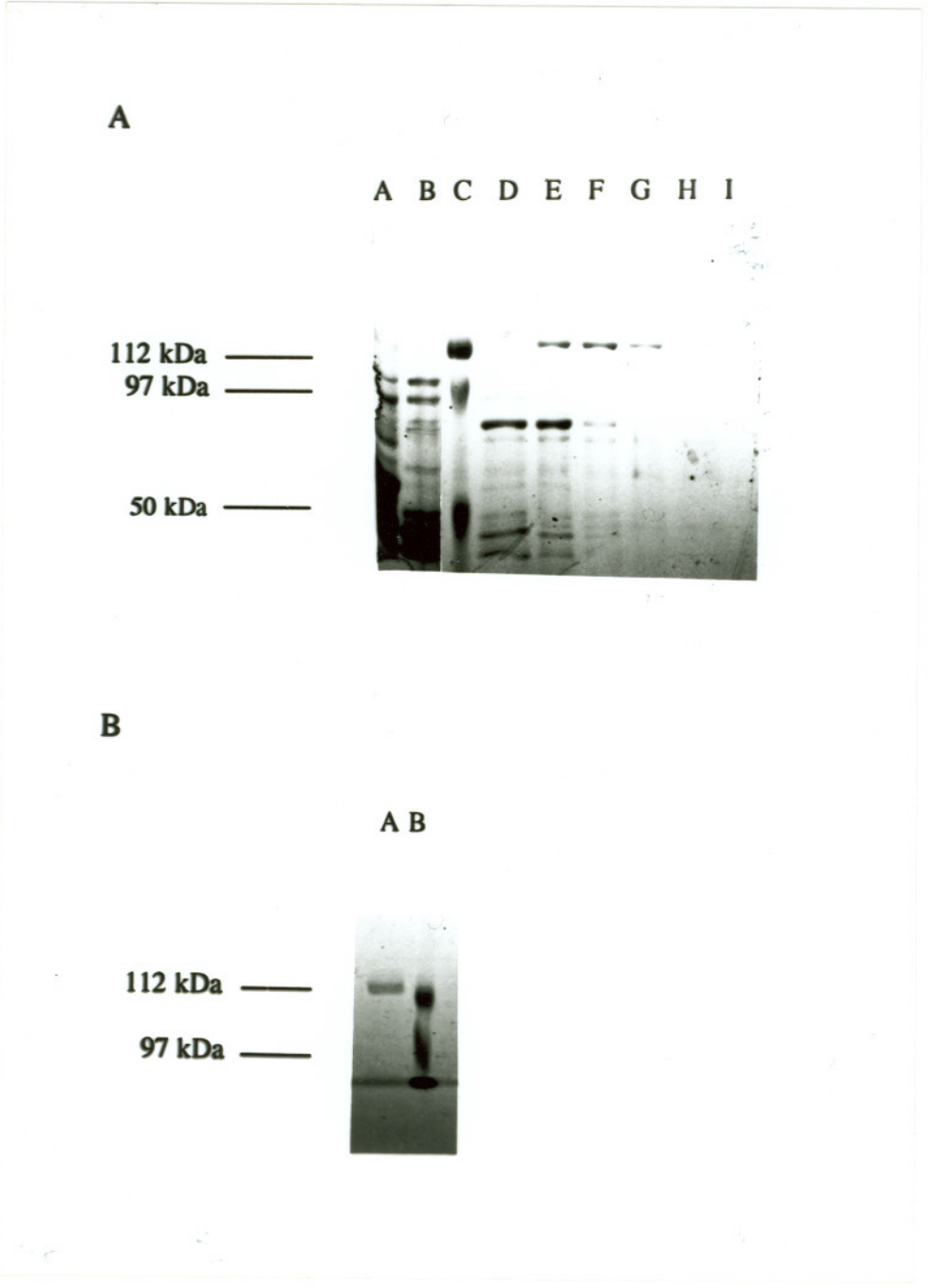

Figure 3.1 **(A)** SDS-PAGE analysis of DEAE-cellulose column-purified 110-kDa protein from SMP. *Lane A,* beef heart submitochondrial particle extract; *lanes B and D–H,* the first and second through seventh fractions, respectively, washed by the column buffer; *lane C,* pre-stained molecular weight marker (2–3 μg/lane). **(B)** SDS-PAGE analysis of the protein sample electrophoresis-eluted from 110-kDa band gel slices. *Lane A,* protein sample after electrophoretic elution; *lane B,* pre-stained molecular weight marker (2–3 μg/lane).

the 110-kDa proteins with high Na^+/Ca^{2+} exchange activity remained in the wash fractions of the DEAE-cellulose column elutions as before. Likewise, a 70-kDa protein, demonstrated by W. Li et al. (1992) to be the mitochondrial heat shock protein hsp 70, also co-purified with the 110-kDa proteins.

3.3 Concentration of the 110-kDa Protein by HA Chromatography

Improvement of the initial ion-exchange chromatography of Na^+/Ca^{2+} protein was sought by trying, in various combinations and orders, DEAE-cellulose, phosphocellulose, and HA columns. The effects of different buffers (TES, HEPES, Tris, and phosphate) of varying pH over the range from 6 to 8, were also examined. By studying the work of others on purification of membrane proteins using HA chromatography with SDS (Horigome et al., 1989) and on the initial protein separation by HA with SDS (Moss et al. 1972), we found after considerable experimentation that an HA column, eluted with a phosphate buffer at pH 6.4 introduced after the initial fractionation on DEAE-cellulose, yielded a very significant improvement in the confinement of the 110-kDa protein to a small number of low-volume fractions. Previously the 110-kDa protein, eluted from the DEAE-cellulose column in the wash fractions, was of very low concentration. This was always a problem in subsequent steps, because necessary concentration of the sample resulted in a parallel problematic increase in detergent concentration. The use of a HA column solved this problem: the large volume of wash from the second DEAE-cellulose column, which contained the 110-kDa proteins, could be passed through a relatively small HA column, and the 110-kDa proteins could then be eluted in just three 1.5-ml fractions of 400 mM $NaPO_4$. From SDS-PAGE and western blot, a very strong band at 110-kDa can be seen (Figure 3.2 A, B) due to this concentration step.

Moreover, after trying several different detergents (Triton X-100, SDS, CHAPS), it was found that replacing Triton with SDS in the HA fractionation significantly increased total protein recovery. There was, unfortunately, a trade-off; to achieve higher protein recovery, it was necessary to increase the SDS concentration

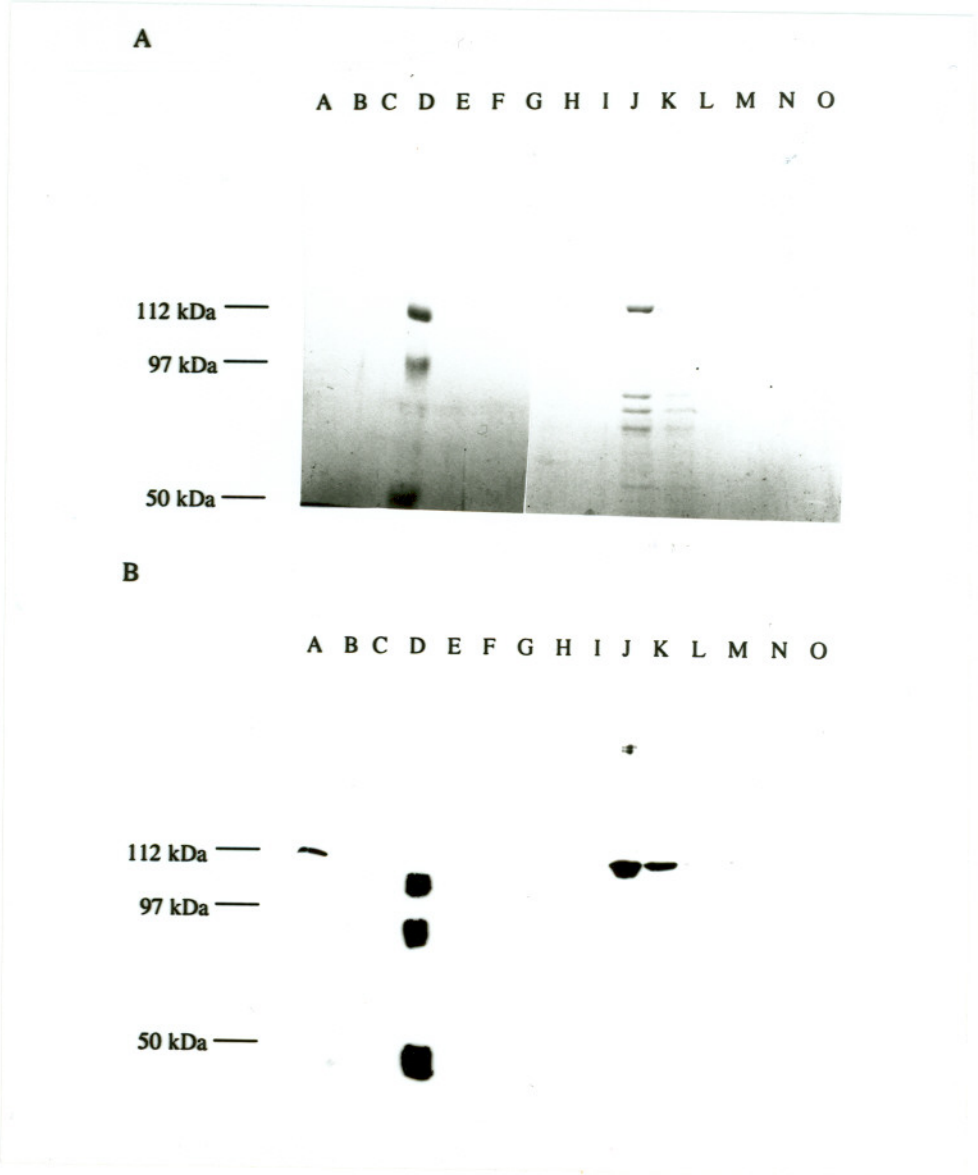

Figure 3.2 SDS-PAGE and western blot (with 110-kDa protein antibody) of fractions from the HA column. *Lane A,* sample before the column; *lane B,* fraction washed by column buffer; *lanes C and E–H,* eluates by 100, 150, 200, 250, and 300 mM $NaPO_4$; *lanes I–L,* the first, second, third and fourth fractions, respectively, of eluates by 400 mM $NaPO_4$; *lanes M–O,* the first, second and third fractions of eluates by 500 mM $NaPo_4$; *lane D,* pre-stained molecular weight marker (2–3 μg/lane). **(A)** SDS-PAGE for HA column analysis. **(B)** Western blot analysis of the duplicated protein pattern of (A), with the 110-kDa protein antibody (titer 1:1000).

to 0.2%, a level which resulted in its precipitation when an attempt was made to purify the protein at 4°C. It was decided that the fractionation using 0.2% SDS could be safely conducted at room temperature provided that it proceeded expeditiously and in the presence of protease inhibitors (pepstain A, leupeptin, and PMSF). Although the use of SDS and anti-proteases would generate problems in later steps of the purification (see below), it was decided that this was a manageable complication justified by gains in both protein yield and improved resolution.

3.4 Electrophoresis Separation of the 70-kDa Protein from the 110-kDa Proteins

Although Cheng (1995) reported that a contaminating 70-kDa mitochondrial membrane heat shock protein could be removed by alkaline extraction prior to column chromatography, this contaminant could never be completely eliminated by this technique. As the complete elimination of all but 110-kDa proteins is crucial to the success of the immuno-affinity purification procedures followed here, the removal of the 70-kDa protein became very important. SDS-PAGE analysis revealed the same fractionation profile for 70 and 110-kDa proteins after both the DEAE-cellulose and the HA columns, and it proved impossible to separate the two bands by the aforementioned variations in the chromatographic methodology. For this reason, a new approach was introduced, based on the electrophoresis and elution of the 110-kda protein.

The separation of the 70 and 110-kDa proteins by electrophoresis is complete, and this procedure has the additional advantage in that it is possible to process relatively large amounts of protein in a single step with better than 90% recovery. Figure 3.1 B demonstrates the results of this procedure, which yield a single protein band at 110-kDa devoid of the hsp-70 contaminant.

3.5 Sample Preparations for the Immuno-Affinity Column

Cheng (1995) was the first to introduce the use of a suitably prepared immuno-affinity column to "negatively" purify the Na^+/Ca^{2+} protein by removing the contaminating TH protein from the 110-kDa protein mixture. As the intention was to submit the purified Na^+/Ca^{2+} protein for sequencing, the final product had to conform to rather severe constraints established by the sequencing facility, namely: sample volume must be no greater than 0.5 ml; detergent (Triton X-100) concentration could not be greater than 1%; and, of course, there needed to be sufficient total protein (at least 50 μg) free of all protease inhibitors. In addition, the proper function of the affinity column required that the 110-kDa protein in the second DEAE column washing fractions (containing 1% Triton-100) be concentrated while keeping detergent concentration constant.

As the DEAE column fractions were concentrated by stirred-cell ultrafiltration, the problem of the concurrent increase in Triton concentration was originally solved by employing a detergent-absorbing gel. Unfortunately, this method resulted in intolerable loss of protein. In the procedures developed here, this problem was solved by the introduction of an HA column between the DEAE and immuno-affinity column steps. This required the substitution of SDS for Triton in order to effectively elute the protein from the HA column. But, since SDS interferes with the immuno-affinity column, it became necessary to re-substitute Triton for the SDS after eluting the now-concentrated 110-kDa proteins from the HA column.

Because these are hydrophobic integral membrane proteins that precipitate without detergent, it was necessary to effect the change of detergents by dialysis. Moreover, as SDS in phosphate buffer will precipitate at 4°C, it was necessary to conduct the detergent exchanges and HA concentration procedures at room temperature. Because the equilibration of the detergents is slow (due to the large micelle size), dialysis was conducted over a four-day period. To protect against bacterial degradation, 0.05% NaN_3 was induced in the HA elution and in the dialysis

buffers. SDS-PAGE analysis of the protein sample before and after these procedures indicated only minor loss of protein and a single 110-kDa band (data not shown).

3.6 Separation of the 110-kDa TH Protein from the Na^+/Ca^{2+} Antiporter by Immuno-Affinity Chromatography

3.6.1 Expression of the transhydrogenase antigen

As shown in Figure 3.3 B, SDS-PAGE analysis of the cell lysates of different constructs showed a distinctively large amount of protein at 39-kDa, the predicted size of expressed product. This is present only in *lane C*, which was the IPTG-induced clone pQE30-TH1, and does not appear in any of the other induced constructs nor in the uninduced strain [data not shown; see Cheng (1995) for details]. Western blot analysis (Figure 3.3 A) shows that only the proteins at 39 kDa in the pQE30-TH1 cell lysates was recognized by the 110-kDa-specific antibodies. These results indicated that none of the *E. coli* bacterial protein was recognized by the 110-kDa antibodies. As this antibody is a mixture of antibodies specific to both the TH and Na^+/Ca^{2+} antiporter proteins, we can infer that the bacteria does not contain any proteins that share antigenicity with either the TH or the Na^+/Ca^{2+} antiporter proteins. Thus, we were confident that only the expressed protein would be recognized by the 110-kDa antibody and that it would not contain the Na^+/Ca^{2+} antiporter.

3.6.2 Polyclonal antibody against the expressed TH protein

The TH protein is a major "contaminant" of the partially purified Na^+/Ca^{2+} protein fraction. Thus, for each 100 μg of protein loaded onto the immuno-affinity column, it was deemed prudent that there be at least a 20-fold (w/w) ratio of antibody to antigen. The idea of passing the sample through the column more than twice proved unworkable, as discussed in **3.6.3** below. Thus it was necessary to prepare approximately 5–7 mg of antibody. The preparation of the partially expressed 39-kDa TH protein, used as the antigen for preparation of the TH antibody, was successfully accomplished with the help of Dr. Hongfa Zhu of our lab. SDS-PAGE analysis of

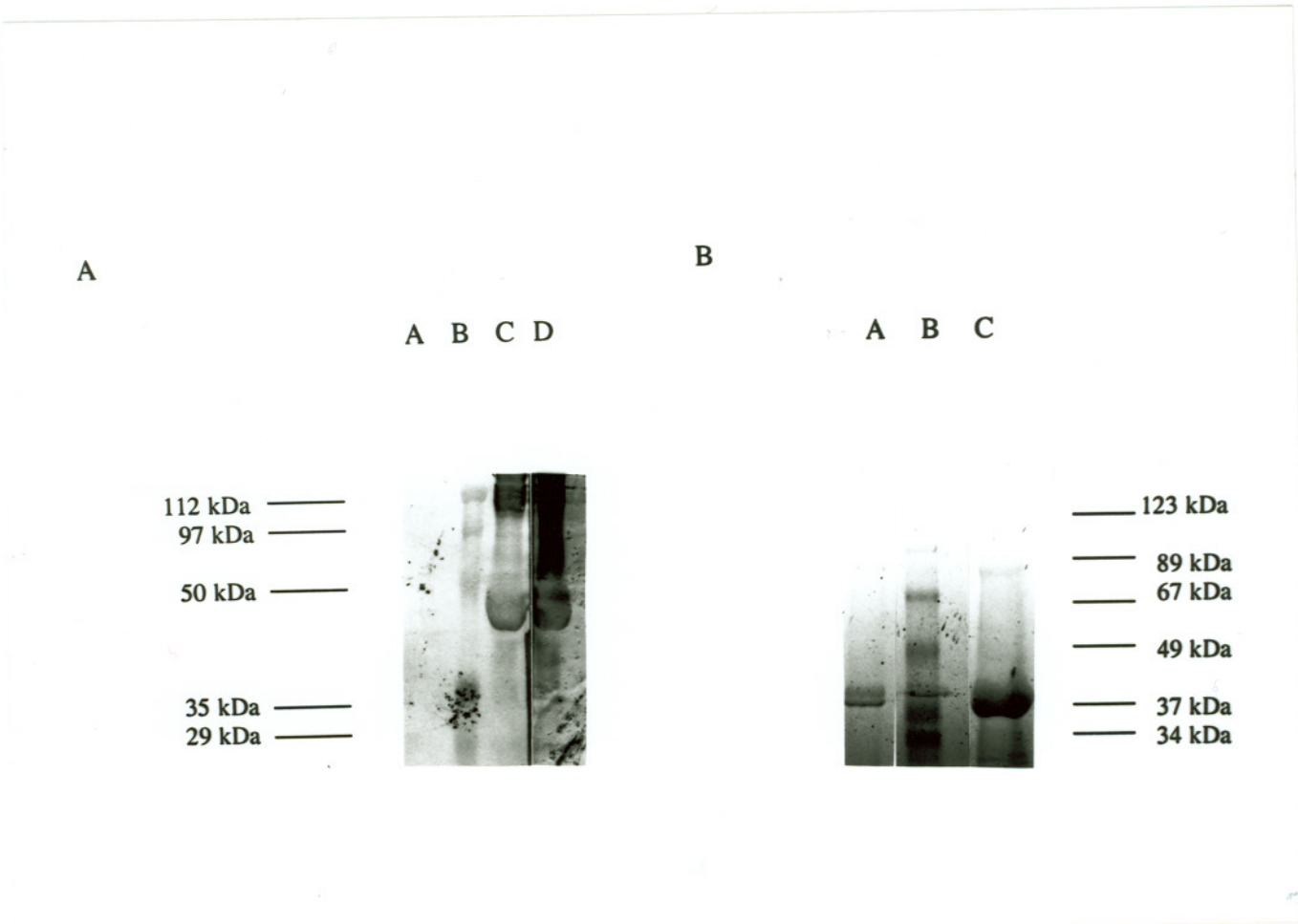

Figure 3.3 Expression of TH antigens in the induced pQE30-TH construct. **(A)** Western blot with 110-kDa antibody of titer 1:400. *Lanes A and C,* uninduced and induced cell of pQE30-TH1; *lane B,* pre-stained molecular weight marker (1–2 μg/lane); *lane D,* western blot for testing newly prepared TH antibody by the known partially expressed TH protein. **(B)** SDS-PAGE analysis of the duplicated protein pattern as (A) (except *lane D*).

the expressed protein showed a band at 39-kDa, and the western blot showed this protein to be recognized by the TH antibody (Figure 3.3 A, *lane C*).

In the process of concentrating the antibody and in getting the TH antibody to bind to the affinity column, a precipitate was observed in solution. The antibody suspension was centrifuged, and SDS-PAGE analysis of both supernatant and precipitate revealed a 50–60-kDa band, believed to be IgG fragments (see Figure 3.4 A). The precipitate may be the result of an antibody–antigen complex (Alberts et al., 1989), formed during the antibody purification procedures by the extremely low pH 2.8 of the elution buffer. The western blot result shows that the TH antibody reacted specifically with the expressed TH protein (Figure 3.3 A, *lane D*). Quantitative protein analysis revealed that this precipitate accounted for less than 10% of total antibody protein, thus it could be discarded and its loss would not adversely affect the immuno-affinity column procedures.

3.6.3 Preparation of the immuno-affinity column

The proper preparation of the column is a critical step in the immuno-affinity chromatographic purification procedure. Initially, the binding efficiency of the agarose group of the column to the antibody was less than 20%. A series of trial experiments to identify the cause focused on such factors as the buffer, the antibody, the reducing agent, the column agarose gel, and incubation conditions. It was finally determined that incubation temperature and time were the most critical factors affecting the coupling efficiency. When the incubation temperature was increased from 4°C to room temperature and the incubation allowed to proceed overnight with shaking, the coupling efficiency increased to over 80%.

The eluting solution (0.1 M glycine, pH 2.8, with 0.2% reduced Triton) was used to wash the column. After antibody binding, the excess IgG, which is loosely bound to the agarose group, was removed to prevent it from contaminating the Na^+/Ca^{2+} antiporter protein in the flow-through fraction. According to the supplier of the Ag/Ab immobilization kit (Pierce), the column can be re-used several times, but our experience suggests otherwise. The reason is perhaps that repeated exposure

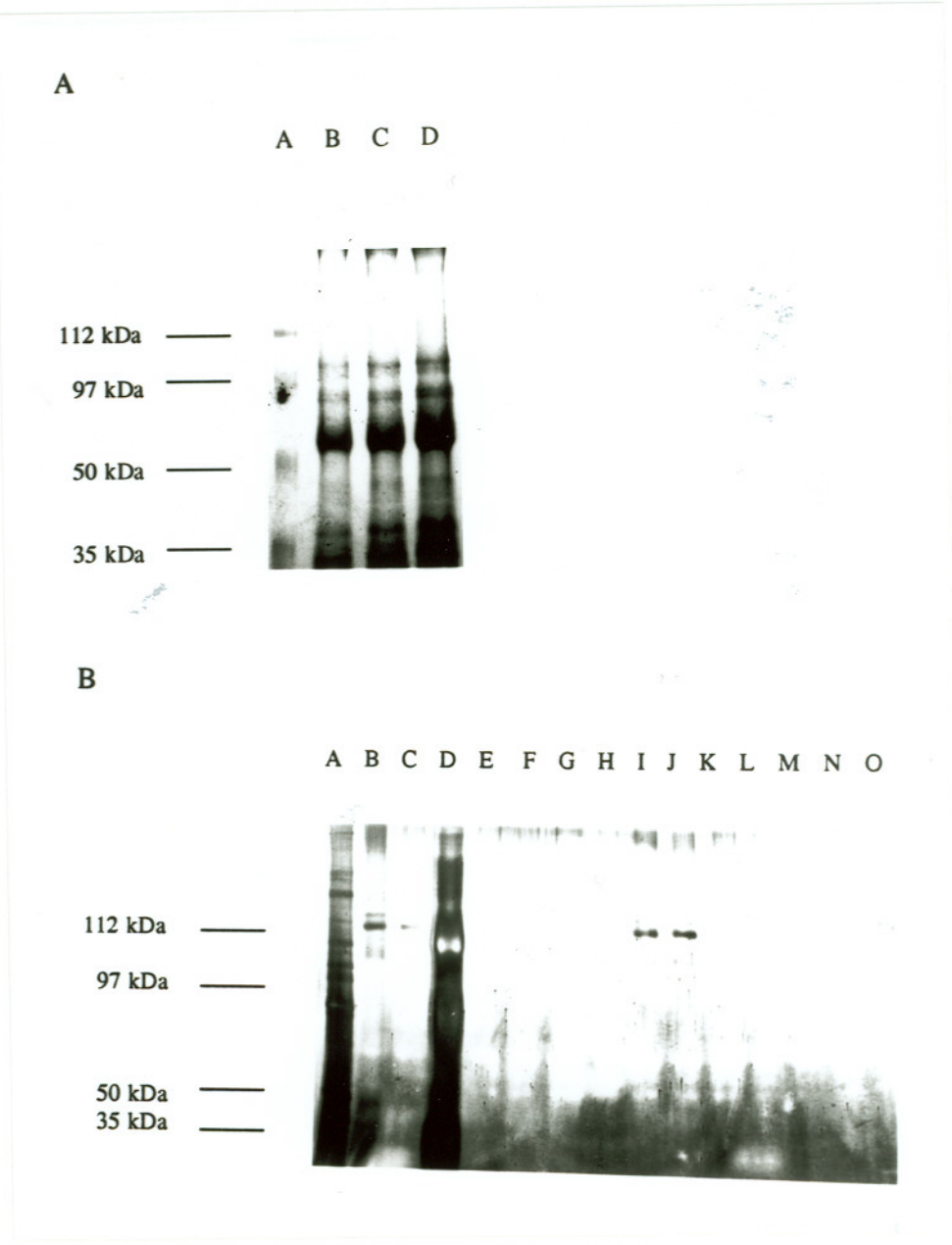

Figure 3.4 **(A)** SDS-PAGE analysis of the suspended precipitate in the TH antibody. *Lane A,* pre-stained molecular weight marker; *lane B,* 10 μl of supernatant; *lanes C and D,* 3 and 5 μg of precipitate, respectively, dissolved in sample buffer. **(B)** SDS-PAGE (silver-stained) analysis of the affinity column fractions. *Lane A,* partially expressed 39-kDa TH protein; *lane B,* flow-through fraction after second pass-through purification; *lanes C, E and F,* the first, second and third fractions, respectively, washed by column buffer in the first pass-through purification; *lanes G–I,* the second, third and fourth eluate fractions by pH-2.8 glycine buffer in the first pass-through purification; *lanes J–L,* the first, second and third fractions washed by the column buffer in the second pass-through purification; *lanes M–O,* the second, third and fourth eluate fractions, respectively, by pH-2.8 glycine buffer in the second pass-through purification; *lane D,* the pre-stained molecular weight marker (0.1–0.5 μg/lane).

to the eluting solution (0.1 M glycine with 0.2% reduced Triton), which has a low pH of 2.8, may alter the secondary structure of the bound antibody. There could also be an extremely adverse effect on the antigen–antibody binding.

3.6.4 Results of the immuno-affinity purification

The gels for the western blots, using either the expressed TH protein antibody or the enriched Na^+/Ca^{2+} antiporter antibody, each contained the same amount of expressed TH protein as control for the ECL development process. Comparison of the two western blots, "normalized" by the TH protein as control, revealed that the antibody against the enriched Na^+/Ca^{2+} antiporter reacted more strongly with protein in the flow-through and wash fractions from the affinity column (theoretically the enriched Na^+/Ca^{2+} antiporter) than did the antibody against the expressed TH protein to these same fractions (Figure 3.5 A, B). In the western blot using the enriched Na^+/Ca^{2+} antibody, the signal of the 110-kDa band in the flow-through fraction is much stronger than in the eluate fractions (Figure 3.5 A). On the other hand, the 39-kDa control band (expressed TH protein) reacted to the TH antibody more strongly than it did to the antibody against the enriched Na^+/Ca^{2+} protein. Thus, any artificial effects (Ab titer, exposure time) due to differing amounts of antibody or to procedural variation can be eliminated. In the western blot using the TH antibody, the signal of the 110-kDa band in the flow-through fraction is also much stronger than the eluate fractions (Figure 3.5 B). From Figure 3.5 A and B, the 110-kDa band protein in the eluted fractions (ideally, mainly TH protein) reacted more strongly to the enriched Na^+/Ca^{2+} protein antibody than to the TH antibody.

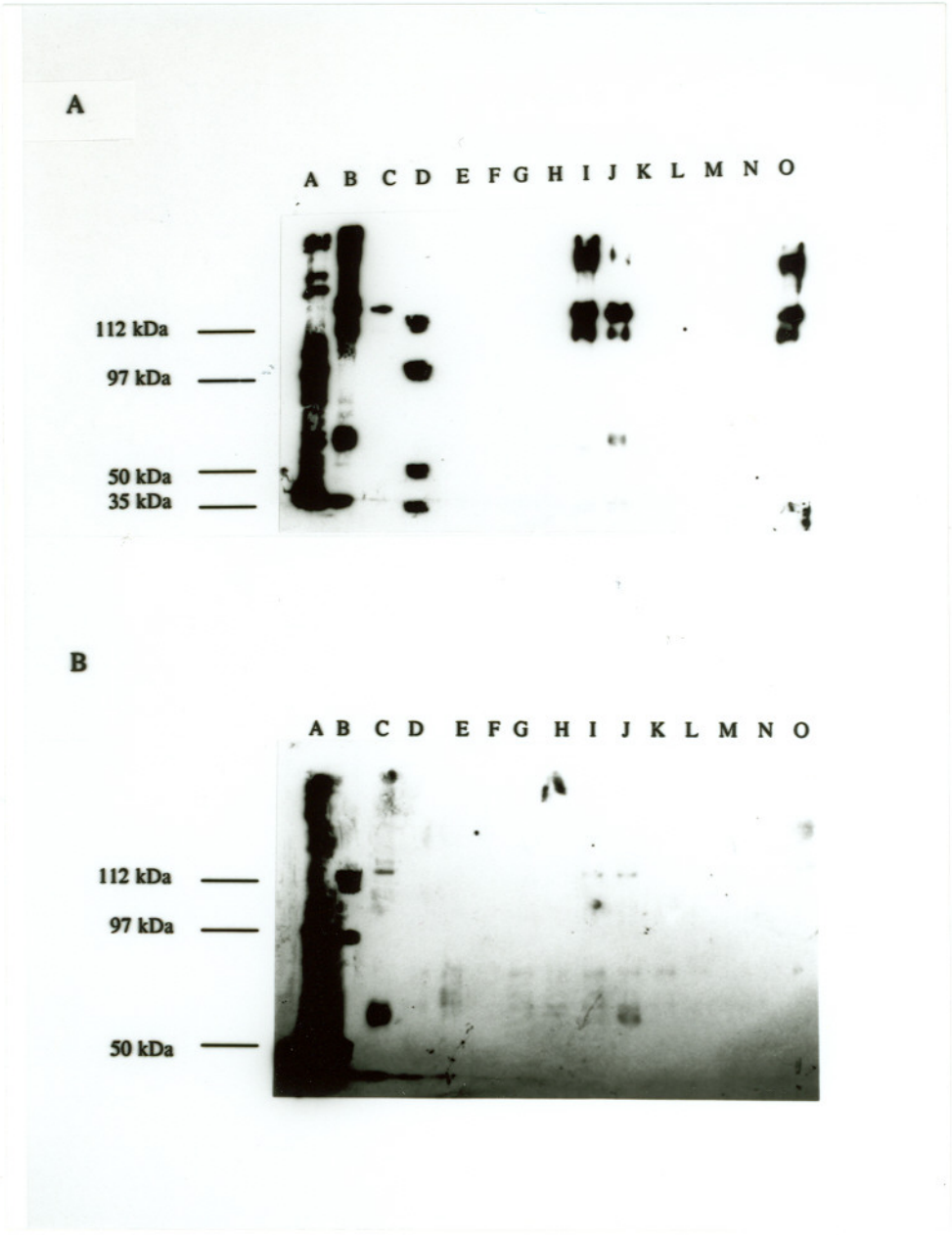

Figure 3.5 Western blot analysis of affinity column fractions. **(A)** Enriched Na^{+}/Ca^{++} (antibody titer 1:400). *Lane A,* partially expressed 39-kDa TH protein; *lane B,* flow-through fraction after second pass-through purification; *lanes C, E and F,* the first, second and third fractions, washed by the column buffer in the first pass-through purification; *lanes G–I,* the second, third and fourth eluate fractions by pH-2.8 glycine buffer in the first pass-through purification; *lanes J–L,* the first, second and third fractions washed by the column buffer in the second pass-through purification; *lanes M–O,* the second, third and fourth eluate fractions, respectively, by pH-2.8 glycine buffer in the second pass-through purification; *lane D,* the pre-stained molecular weight marker (0.1–0.5 μg/lane). **(B)** TH antibody (titer 1:500), the duplicated protein pattern as (A), except that *lane C* is the pre-stained molecular weight marker.

CHAPTER 4
DISCUSSION

4.1 Background of this Work on the Purification of the Na^+/Ca^{2+} Antiporter

Much of our motivation to pursue purification and eventual sequencing of the mitochondrial Na^+/Ca^{2+} antiporter derives from the success of Philipson et al. (1988) in sequencing the Na^+/Ca^{2+} antiporter from the sarcolemmal membrane. Following their work, we have adopted the use of non-ionic detergents and ion-exchange (DEAE-cellulose) chromatography as the central approach to the isolation and purification of the mitochondrial Na^+/Ca^{2+} antiporter. Our initial approach—monitoring our progress through functional assays of reconstituted purified fractions—led to a 110-kDa protein which, upon cloning, was found to contain TH (Cheng, 1995) as a major contaminant to the Na^+/Ca^{2+} protein. The focus of my work has been the development and refinement of techniques and procedures, utilizing the methods of molecular biology and immuno-affinity purification, to separate the Na^+/Ca^{2+} antiporter protein from the TH contaminant in the 110-kDa protein mixture.

A review of the progress made in the purification of the mitochondrial Na^+/Ca^{2+} protein—first begun by W. Li et al. (1992) and continued by Cheng (1995)—can be summarized by the following facts: (i) both the TH and Na^+/Ca^{2+} proteins have the same molecular weight and very similar electrical charges; (ii) both are highly hydrophobic integral membrane proteins which likely form mixed detergent micelle complexes during solubilization; (iii) SDS interferes with the functioning of the immuno-affinity column used to separate the two proteins as well as enzyme digestion procedures needed in sequencing; (iv) there is no effective specific binding ligand, such as an inhibitor, toxin or affinity label, that can be used to identify the

purified protein; (v) the polyclonal antibodies, employed as a means to follow purification, were raised against a 110-kDa protein mixture containing both proteins, and thus a distinction cannot be made between them; (vi) functional assays are inconclusive because the contaminant TH protein cannot be eliminated, and negative results can be attributed to a failure in technique.

4.2 Progress in Purification of the Na^+/Ca^{2+} Antiporter

Cheng (1995) reported the existence of two proteins in the 110-kDa band—the Na^+/Ca^{2+} antiporter and the TH protein. Previously, purification consisted of just two steps: DEAE-cellulose chromatography followed by "negative" purification of the Na^+/Ca^{2+} antiporter by an affinity column coupled to TH antibody. The fraction from the DEAE-cellulose column was of large volume and the Na^+/Ca^{2+} antiporter was very dilute (~ 50 μg/ml). This fraction was also known to contain another protein of about 70-kDa. As the immuno-affinity column can only be used with very small volumes, the sample needed to be concentrated. And the 70-kDa band needed to be removed, preferably prior to the affinity column; otherwise it would persist as a contaminant to the Na^+/Ca^{2+} protein in the affinity column flow-through. Cheng (1995) used stirred-cell ultrafiltration to concentrate the DEAE column fraction about 50-fold, but in the process the Triton X-100 detergent concentration increased to over 12%. Because the immuno-affinity column cannot function properly at such high detergent concentrations, a detergent-absorbing gel was used to reduce it to 1%. Unfortunately, this method of removing the Triton X-100 led to inconsistent results and to a prohibitive loss of protein.

After trying several different ion-exchange resins and various detergents, the combination of SDS and HA gave a dense well-focused band and eluted the 110-kDa protein in a small number of fractions (see Figure 3.2 A,B). The SDS/HA combination allowed us to concentrate the protein without the problems of high detergent concentrations or loss of protein.

As sequencing of the final product was the rationale of the purification efforts, ultra-pure SDS and reduced Triton-100 were used in the HA column and SDS-to-Triton dialysis procedures, respectively, to decrease the possibility of sequencing signal loss due to amino acid blockage by formly acetyl and pyroglutamyl reactants (Hirano et al., 1992).

The band patterns from the HA column were the same as those from the DEAE-cellulose (compare Figures 3.1 A and 3.2 A). The HA column apparently has no separating function with respect to the TH and Na^+/Ca^{2+} proteins; neither does it separate the 110-kDa and 70-kDa proteins. Thus additional purification was required.

Removal of the 70-kDa protein was accomplished by running the appropriate HA eluting fraction on a preparative SDS-PAGE, excising the 110-kDa band, and recovering it from the gel slice by electrophoretic elution. This led to a single 110-kDa protein band (Figure 3.1 B) for immuno-affinity column separation of the TH and Na^+/Ca^{2+} proteins.

When the 110-kDa protein mixture (in 0.2% SDS) was applied to the TH-antibody affinity column directly after its elution from the gel, the affinity column failed to bind the TH component in the 110-kDa protein (results not shown). While the exact cause is not known, SDS likely interferes with antibody recognition of the TH protein, possibly through denaturing effects on the TH antibody.

The exchange of SDS for Triton proved to be a major problem. From previous experience, once such hydrophobic proteins are allowed to precipitate, they are nearly impossible to re-solubilize without making them unrecognizable to the TH antibody. Thus, dialysis was employed as a means to simultaneously remove the SDS and introduce Triton. As SDS protein micelles can be as "large" as 20-kDa (Findlay, 1989), a rather porous dialysis membrane was required. Moreover, because the equilibration of Triton is substantially slower than SDS (the Triton-X 100 micelle is about 90-kDa), 0.2% Triton (v/v) was added to both the sample and the dialysis buffer to ensure the continual presence of detergent as the SDS was removed. The choice of a 0.2% Triton X-100 concentration was so that, after a five-fold concentration of the collected flow-through from the affinity column, the sample

would contain 1% Triton, the upper limit for the sequencing procedures. PBS, the recommended affinity-column buffer, was chosen as the dialysis buffer. It was found, however, that SDS has very low solubility in PBS at lower temperatures (4°C). Therefore, the dialysis was done over a period of four days at room temperature, with appropriate precautions taken to avoid protease and bacterial degradation of the proteins. Even with this prolonged dialysis, there still appeared a gelatinous precipitate of unknown composition in the Triton-solubilized sample after its concentration.

Two "parallel" western blots were performed on the results of the affinity-column, i.e., on the column flow-through and on the column eluates, which in principle contained only the Na^+/Ca^{2+} or the TH proteins, respectively. Included on each blot was an equal amount of expressed TH protein as a "control" to assistant in estimating the relative titer of the antibodies used.

One blot was reacted with the so-called "enriched" Na^+/Ca^{2+} antibody [previously prepared by Cheng (1995); see **2.2.5.2**], and showed a strong signal against the column flow-through and a relatively weak signal against the eluate. This indicated either that there remained some residual Na^+/Ca^{2+} protein in the column eluate (even after the extensive pre-elution washing) or that there yet remained some TH antibody in the "enriched" Na^+/Ca^{2+} antibody preparation, or both. In either case, this result indicates a definite, if not total, separation of the two 110-kDa proteins from each other and shows that the immuno-affinity purification procedures developed in this work represent significant progress in the overall quest to separate the Na^+/Ca^{2+} protein from this TH contaminant.

The result of the second western blot, reacted with the expressed-TH antibody, unfortunately failed to corroborate the results of the first western blot. The signal against the eluate was not stronger than that against the flow-through. Indeed, when a possible difference in antibody titer (via the "control" protein on each blot) was taken into account, this second western blot indicated that the column eluate contained even less TH protein than did the Na^+/Ca^{2+}. This result, while it does not contradict that of the first western blot, would seem to indicate that more refinement of the

procedures is required before the immuno-affinity purification technique can be relied upon to accomplish the separation objective. Overall, the approach appears to work to some degree, but additional time and resources appear to be required to make it viable.

4.3 Immuno-Affinity Chromatographic Purification of the $Na+/Ca^{2+}$ Antiporter

Ideally, the immuno-affinity purification procedure should completely remove the TH protein "contaminant" from the 110-kDa protein mixture applied to the TH-antibody affinity column. The TH protein would be retained, while the Na^+/Ca^{2+} protein should pass freely through the column and appear (exclusively) in the flow-through. Thereafter, following extensive washing, the TH protein itself could be eluted from the column. SDS-PAGE analysis of these two fractions would, of course, show the characteristic 110-kDa band for both (possibly varying in the relative amount of protein in each). The distinctive result would be observed in the western blot analysis of the two fractions, each reacting exclusively to the antibody specific for the protein (Na^+/Ca^{2+} or TH) it contained. In reality, the actual results of the western blots were not as definitive as one would have hoped.

As discussed above, both affinity column fractions (the flow-through and the elution) were recognized by both antibodies in such a way as to render the results inconclusive. The western blot using the "enriched" Na^+/Ca^{2+} antibody indicated definite, though not complete, separation of the Na^+/Ca^{2+} protein from the TH contaminant. The enriched Na^+/Ca^{2+} antibody signal in the flow-through was approximately ten times stronger than this fraction's signal to the expressed TH antibody (corrected for difference in antibody titer). Paradoxically, the reaction signals for the eluted fraction also showed this same pattern—the signal of the enriched Na^+/Ca^{2+} antibody was also greater than that for the TH antibody. The obvious explanation—that the titer or "purity" of the two antibodies differ (or, for that matter, that the reactivity of the antibody conjugate used in the ECL visualization of

the signals is different)—is not plausible when one compares the signals of the expressed-TH protein "control" on each blot (see Figure 3.5 A and B, *lane A*).

A more likely explanation lies in the consideration of the interaction of the detergent micelle–protein complex with the affinity column. The micelles contain a mixture of both TH and Na^+/Ca^{2+} proteins (which is why it is so difficult to effect separation in the first place). As this mixture passes through the affinity column, the TH antibody attached thereto binds to the TH protein in the micelle complex in such a manner that the micelle cluster itself is retained by the column. In this way, a significant amount of the Na^+/Ca^{2+} protein could also be retained in the column, only to be eluted with the TH in the final step. Mixed protein micelles are known to behave in very complex ways, undergoing various aggregations and transitions which are strongly determined by factors such as the pH, temperature, and ionic strength of their environment. Thus, it is not inconceivable that the PBS washing and low-pH elution of the column could be, in part, responsible for the paradoxical western blot results.

But why then is the TH antibody signal so weak in the eluted fractions, especially as the TH protein is the dominant component of the 110-kDa protein mixture? It is possible that the column elution failed to removed the bound TH protein so that a significant amount remained on the column. Alternatively, the TH protein may have been less "stable" than the Na^+/Ca^{2+} protein, or otherwise may have undergone relatively more degradation during the prolonged periods required for dialysis. Indeed, notwithstanding the use of protease and bacterial inhibitors to protect the protein mixture, it may be simply that the TH protein is intrinsically more labile than the Na^+/Ca^{2+} protein. But, as it is impossible to separately quantitate the relative amounts of the TH and Na^+/Ca^{2+} proteins in the 110-kDa mixture, this explanation remains speculative at best.

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BIOGRAPHICAL SKETCH

The author was born on November 10, 1957, in Beijing, the People's Republic of China. In 1978, she began her undergraduate education in the Department of Medicine, Nantong Medical College, Jiangsu Province, and in 1982 received her Bachelor of Medicine degree. She then accepted a position as a resident physician at the Nantong Medical College Affiliated Hospital. In the fall of 1986, the author began her graduate studies in medicine at the Nanjing Railway Medical College, receiving a Master of Medicine degree in 1989. Thereafter she held a position as resident physician at the Nanjing Railway Medical College Affiliated Hospital.

In January 1994, she began graduate research studies in the Department of Chemistry, Biochemistry and Molecular Biology at the Oregon Graduate Institute of Science and Technology under the supervision of Dr. Keith D. Garlid, where she worked on the purification and sequencing of the mitochondrial Na^{+}/Ca^{++} antiporter.