Immunological Separation of Mitochondrial Na^{+}/Ca^{2+} Antiporter Protein

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ABBREVIATIONS

ATP	adenosine triphosphate
CIP	calf intestinal alkaline phosphatase
CMC	critical micellization concentration
DTT	dithiothreitol
DEAE	*O*-diethylaminoethyl
DHFR	dihydrofolate reductase
EGTA	ethyleneglycol-bis-N,N'-tetra-actic acid
IPTG	isoprophy-β-D-thiogalactoside
NAD	nicotinamide adenine dinucleotide
NTA	nitrilo-tri-acetic acid
SBFI	sodium-binding benzofuran isophthalate
SDS-PAGE	sodium dodecyl sulfate-polyacrylammonium gel electrophoresis
SMP	submitochondrial particle
TEA^+	tetraethylammonium cation
TES	N-tris(hydroxymethyl)methy-2-aminoethenesulfonic acid
TH	mitochondrial ticotinamide nucleotide transhydrogenases
TPP^+	tetra-phenylphosphonium

Abstract

Immunological Separation of Mitochondrial Na^+/Ca^{2+} Antiporter Protein

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The Na^+/Ca^{2+} antiporter plays a key role in regulating matrix Ca^{2+}, which coordinates cell energy demand and mitochondrial ATP production. The Na^+/Ca^{2+} exchange activity was identified with a 110-kDa mitochondrial inner membrane protein that catalyzed Na^+-dependent Ca^{2+} transport and Ca^{2+}-dependent Na^+ transport (Li et al., 1992). Using 110-kDa protein specific polyclonal antibody as probe to screen a bovine heart λgt11 cDNA library, the selected positive clones were verified as to mitochondrial nicotinamide nucleotide transhydrogenases (TH). This result indicated that the 110-kDa protein band contained transhydrogenase contamination in addition to the putative Na^+/Ca^{2+} antiporter, and that TH occupied a significant portion of the 110-kDa protein. Therefore, the 110-kDa protein specific antibodies were also mixtures of antibodies to the Na^+/Ca^{2+} antiporter and to TH. To separate TH contamination and identify the Na^+/Ca^{2+} antiporter, molecular biological techniques were applied. A partial TH antigenic peptide was excised from a TH clone and was then successfully expressed in *E. coli*. TH antibodies against this antigen were further immunopurified. With these authentically pure antigens and antibodies available, primary separation was realized by the Rotofor-Cell technique, which showed that there were two proteins of pI 8.1 and 9.2 in the 110-kDa region. The protein with pI 9.2 was only recognized by the mixed antibodies to the 110-

kDa protein, but not by the antibodies against the expressed TH antigen. In contrast, the protein with pI 8.1 was recognized by both antibodies. To remove TH from Na^+/Ca^{2+} antiporter in the 110-kDa protein fraction, antibodies against TH antigen were immobilized on a chromatography column and used to perform negative purification. Putative Na^+/Ca^{2+} protein was significantly enriched in the flow-through fraction; however, TH protein was difficult to remove completely. The antibody specific to the immunopurified 110-kDa protein (enriched putative Na^+/Ca^{2+} protein) showed inhibition of Na^+/Ca^{2+} transport in reconstitutive activity while the TH antibody did not.

Chapter 1
Introduction

1.1 Calcium as a Secondary Messenger in Mitochondria

One of the basic tasks of intracellular free Ca^{2+} is as a second messenger carrying extracellular signals to a large number of biochemical activities in cytosol and in various subcellular compartments. The messenger role of cytosolic Ca^{2+} is amplified by its low ionic concentration, which is very sensitive to the amounts of calcium being mobilized. The precise intracellular regulation of Ca^{2+} depends on the concerted operation of specific Ca^{2+} transport systems located in the plasma membrane, in mitochondria, and in endo(sarco)plasmic reticulum, which transport Ca^{2+} between cells and the environment, and between cytoplasm and organelles.

1.1.1 Intracellular Free Calcium and Mitochondria

In early recognition, mitochondria were regarded as an intracellular Ca^{2+} store, and mitochondrial Ca^{2+} was mobilized by hormones (Joseph & Williamson, 1983). This misconception was because of crude mitochondrial fractions being heavily contaminated with endoplasmic reticulum. For many years the major physiological role of mitochondrial Ca^{2+} was advocated to act as buffer in regulation of cytosolic Ca^{2+} (Carafoli & Crompton, 1978; Scarpa & Graziotti, 1973; Joseph & Williamson, 1983). However, studies in the presence of both mitochondria and endoplasmic reticulum have showed that the extravesicular Ca^{2+} level was determined predominantly by endoplasmic reticulum not mitochondria at physiological conditions (Becker, 1980; Carafoli, 1986). The demonstration that mitochondria handled Ca^{2+} with low affinity (Crompton et al., 1976) and the finding that matrix contained much less free Ca^{2+} in situ than previously assumed

(Carafoli, 1979) have led to a re-evaluation of their importance in the regulation of cytosolic Ca^{2+}.

As interest in mitochondria as cytosolic Ca^{2+} buffer decrease, awareness of their importance as regulators of their own internal Ca^{2+} concentration has increased. Current data indicate that some intramitochondrial enzymes are modulated by oscillations of matrix Ca^{2+} in the physiological μM range. Thus, it is not surprising to find that the action of mitochondrial calcium is related to respiration and ATP production, representing the vital role played by mitochondria.

1.1.2 Intramitochondrial Calcium-Sensitive Enzymes

The role of intramitochondrial free Ca^{2+} has been a mystery for some time. A significant breakthrough in this area came with the discovery that several intramitochondrial dehydrogenase enzymes involved in ATP synthesis are regulated by the physiological concentration of intramitochondrial Ca^{2+}.

1.1.2.1 Mitochondrial Dehydrogenases

In the heart and other mammalian tissues, there are three intramitochondrial dehydrogenases that occupy key regulatory sites in oxidative metabolism that can be activated by increased intramitochondrial free Ca^{2+} concentration within the range of 0.1-10 μM (Denton et al., 1972, 1978; McCormack et al., 1979); they are pyruvate dehydrogenase, NAD^+-isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. This effective matrix Ca^{2+} range is achieved in the presence of physiological concentrations of Mg^{2+}, which inhibits mitochondrial Ca^{2+} uptake, and Na^+, which promotes Ca^{2+} egress from mitochondria (Crompton, 1985; McCormack & Denton, 1987). The activation of these enzymes by Ca^{2+} can be demonstrated in isolated enzymes and intact mitochondria in many different tissues. In both cases, the Ca^{2+} sensitivities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase are similar (K_m in the range of 0.1-1 μM), whereas that of

NAD^+-isocitrate dehydrogenase appears to be less (K_m in the range of 3-30 μM) (Denton et al., 1988). It is also demonstrated that the tricarboxylic acid cycle turnover rate can be activated at Ca^{2+} of 1 μM and this activation process will result in elevated NADH production and ATP synthesis (Denton & McCormack, 1985; Hansford, 1985).

1.1.2.2 Mitochondrial Pyrophosphatase

In addition to dehydrogenases, matrix pyrophosphatase activity can be effectively inhibited by Ca^{2+} concentrations within the same micromolar range as dehydrogenases, with K_i of 0.3 μM (Davidson & Halestrap, 1987); the resulting increase of matrix PPi content is responsible for the parallel increase in matrix volume and total mitochondrial adenine nucleotides content (Davidson & Halestrap, 1987, 1989). Ca^{2+} causes swelling of energized mitochondria through the effect of pyrophosphate by promoting the permeability of K^+ influx (Davidson & Halestrap, 1987, 1989). As a result, the increase in matrix volume stimulates the respiratory chain, and hence, NADH utilization.

1.1.2.3 ATP Synthetase in Mitochondrial Inner Membrane

The other enzyme indicated to be activated by Ca^{2+} along the energy-production pathway is ATP synthetase (Yamada et al., 1980), which is activated by the Ca^{2+}-dependent dissociation of an inhibitory subunit (CaBI) by Ca^{2+} at 1 μM (Yamada & Huzel, 1988). Recently, Das and Harris (1990a, 1990b) observed that ATP synthetase can be reversibly modulated in vivo by association and dissociation of CaBI, with ATP synthetase activity rising with the energy demand imposed by agents such as isoprenaline, and falling when the demand is removed. They also found that, in pathological circumstances, such as hypertension, this regulatory mechanism is defective and may, therefore, be the cause of the deficiency in ATP production within contracting cells (Das & Harris, 1990b).

1.1.3 Mitochondrial Calcium Regulates ATP Production

The regulation by physiological range of calcium on matrix enzymes, which play key roles in mediating oxidative phosphorylation, clearly indicates that Ca^{2+} can affect mitochondrial ATP production. Evidence is accumulating that hormones regulate intramitochondrial oxidative metabolism through alterations in intramitochondrial Ca^{2+} (Denton et al., 1985, 1988; McCormack & Denton, 1985). When hormones and other extracellular agents elicit an increase in cytoplasmic free Ca^{2+} to stimulate energy-requiring processes such as contraction or secretion, they also, as a result, bring about increases in intramitochondrial Ca^{2+} concentration and, hence, increases in oxidative metabolism to meet these enhanced energy demands through activation of intramitochondrial Ca^{2+}-sensitive enzymes (Denton et al., 1985; Hansford, 1985; McCormack,1988). In this way, pyruvate oxidation and citric acid cycle flux are stimulated, and there is an increase in NADH supply for the respiratory chain and an increase in ATP production. Calcium is the only known second messenger for hormones that is transferred across the mitochondrial inner membrane.

The regulation of mitochondrial ATP production by Ca^{2+}, called "extrinsic" control, offers tremendous advantages over the "intrinsic" control-- namely the phosphorylation potential that is shown as ADP/ATP and NAD/NADH concentration ratios. The Ca^{2+}-mediated cellular events, such as extraction and secretion, induced by extracellular agents (hormones etc.), increase the demand for ATP. The passive feedback mechanism by phosphorylation potential seems to be insufficient. The Ca^{2+} signal provides an alternative means for meeting the cellular ATP demand at high energy levels by increasing the supply of substrate for ATP production without the need for large changes in these key metabolite ratios (McCormack,1988). Hence, the Ca^{2+} signal provides an alternative means to meet cellular ATP demands in highly energetic states.

1.2 Mitochondrial Calcium Cycling

1.2.1 The Transport Mechanism

Investigations over the past thirty years have revealed that mitochondria possess a system of considerable sophistication for the transport of Ca^{2+} across their inner membranes. The influx of Ca^{2+} is demonstrated as a carrier (Selwyn et al., 1970) that transfers Ca^{2+} into mitochondria driven by the H^+ electrochemical gradient across the mitochondrial membrane; however, the efflux of Ca^{2+} is mediated by a separate pathway — a distinct carrier for Ca^{2+} efflux that is induced by extramitochondrial Na^+ (Carafoli et al., 1974). In the physiological range, both carriers operate unidirectionally to permit continuous recycling of Ca^{2+} across the inner membrane and, thereby, provide the basis for a kinetic regulation of the distribution of Ca^{2+} between the cytosol and the mitochondrial matrix.

The mitochondrial calcium cycling system in the heart mainly consists of four major components, namely the H^+ gradient, Na^+/H^+ antiporter, Ca^{2+} uniporter, and Na^+/Ca^{2+} antiporter. Mitochondria generate and maintain a proton gradient across the inner membrane by respiration; in addition, its metabolism, volume homeostasis, pH regulation, etc., all depends on this proton gradient. In the case of Ca^{2+} cycling, the proton gradient provides the driving force for the Ca^{2+} uniporter mediated Ca^{2+} influx, and the Na^+/H^+ antiporter which transports Na^+ efflux exchange with H^+ influx is also driven by the inward H^+ gradient. The inward Na^+ gradient mediated by the Na^+/H^+ antiporter provides the driving force to extrude Ca^{2+} out of the mitochondria via the Na^+/Ca^{2+} antiporter. Therefore, the two elements that are directly involved in Ca^{2+} transport in mitochondria are the Ca^{2+} uniporter and the Na^+/Ca^{2+} antiporter.

1.2.2 Calcium Influx

It has been firmly established that Ca^{2+} uptake by mitochondria from a wide range of sources is mediated by a uniporter (Selwyn et al., 1970; Rottenberg & Scarpa, 1974),

which sequesters extramitochondrial Ca^{2+} driven by the membrane potential (~180 mV, negative inside) generated by the mitochondrial electron transport oxidation-reduction proton pumps. This carrier is defined as an electrophoretic uniporter, which sequesters a Ca^{2+} of 2 charges/cycle without coupling the transport of any other ion or molecule.

The striking feature of the Ca^{2+} uniporter is its extremely high turnover rate of over 20,000 s^{-1} for less than 1 pmol/mg (Reed & Bygrave, 1975; Gunter & Pfeiffer, 1990). The uniporter exhibits sigmoidal kinetics with respect to extramitochondrial Ca^{2+} with K_m of at least 10 μM (Reed & Bygrave, 1975), which is much higher than the normal range of free Ca^{2+} concentrations found in the cytoplasm. Moreover, Mg^{2+}, at concentrations likely to be present in the cytoplasm (0.5-2 mM), is known to be a competitive uptake inhibitor (Lenzen et al., 1986). Hence, in physiological conditions, the Ca^{2+} uniporter only operates at its sub-maximum possible velocity.

The energy-linked uptake of Ca^{2+} is inhibited by agents that dissipate the membrane potential generated by respiration, like the uncouplers of oxidative phosphorylation. La^{3+} was found to inhibit Ca^{2+} uptake competitively (Scarpa et al., 1973), while the most often used inhibitor is polycation — ruthenium red which blocks the Ca^{2+} uniporter non-competitively (Reed & Bygrave, 1975). Spermine was reported to activate the Ca^{2+} uniporter and might contribute to the regulation of mitochondrial matrix Ca^{2+} by acting on the Ca^{2+} transport system (Lenzen et al., 1986). It is also observed that α-adrenergic agonists appear to increase the activity of the Ca^{2+}-uptake pathway in both heart and liver mitochondria (Crompton et al., 1985).

The regulation of the Ca^{2+} uniporter can be a very important element for matrix Ca^{2+} activity. However, based on the estimation of cytosolic/mitochondrial ionized Ca^{2+} gradients, it clearly indicates that the Ca^{2+} uniporter operates far from equilibrium. This may suggest that there are other independent mechanisms involved in regulating matrix Ca^{2+}.

1.2.3 Ca^{2+} Efflux Pathway

It has been demonstrated in many tissues that Ca^{2+} release is mediated by a pathway independent of the Ca^{2+} uptake. This efflux mechanism is not due to a non-specific leak of Ca^{2+} from the matrix, since the flux occurs against the gradient of Ca^{2+} electrochemical potential. The transfer of calcium out of mitochondria occurs mainly via direct Na^+-induced Ca^{2+} efflux -- a Na^+/Ca^{2+} antiporter (Carafoli et al., 1974; Crompton et al., 1978). However, mitochondria also contain a Na^+-independent efflux pathway that is particularly evident in liver and kidney mitochondria at higher intramitochondrial calcium loading. The mechanism of this latter pathway is poorly understood, but it is widely believed that it involves direct Ca^{2+}/H^+ exchange (Vaghy et al., 1982). The detail mechanism mediated by the Na^+/Ca^{2+} antiporter is discussed later.

1.2.4 Integration of Ca^{2+} Influx and Efflux Pathways — the Ca^{2+} Cycle

The simultaneous operation of independent influx and efflux pathways across the inner membrane results in the continuous cycling of Ca^{2+} between the cytosolic and matrix compartments. The dynamic balance of Ca^{2+} across the inner mitochondrial membrane is the result of the concerted operation of the electrophoretic uptake route and the Na^+-promoted release pathway (or of the putative H^+/Ca^{2+} exchange system in mitochondria where the Na^+-independent route is predominant). The mitochondrial Ca^{2+} cycle is illustrated in Figure 1.1.

Although the uniporter can take up Ca^{2+} at least ten times faster than the Ca^{2+} released by the Na^+/Ca^{2+} antiporter, normally, mitochondria operate in an ambient Ca^{2+} activity which can be assumed to be <1 μM outside and 1-10 μM inside (Denton, 1978). Moreover, they are presumably surrounded by 1 mM Mg^{2+} to substantially slow down the uptake route (Hutson, 1977), and by ~5 mM ionized Na^+ to stimulate the Na^+-induced Ca^{2+} efflux route (Lee et al., 1975). Therefore, in vivo, the Ca^{2+} uptake route normally operates at only a fraction of its maximum possible velocity, but the Ca^{2+} release route

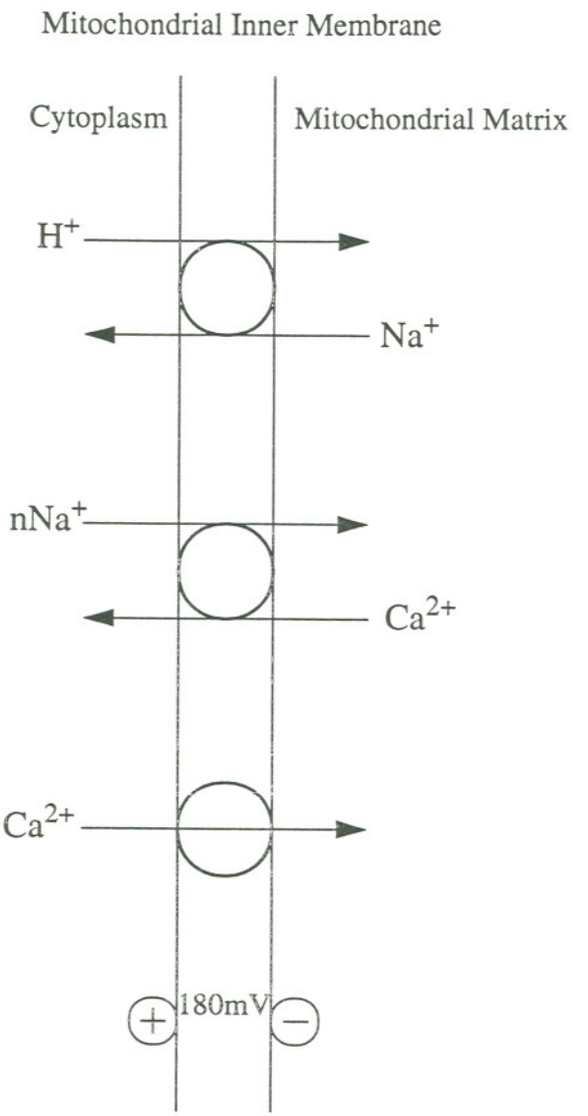

Fig 1.1 The mitochondrial Ca^{2+} cycle. Mitochondria possess separate pathways in the inner membrane for Ca^{2+} influx and efflux. The uptake of Ca^{2+} by mitochondria is mediated by an electrophoretic uniporter that is driven by membrane potential (~180 mV, negative inside). The principal Ca^{2+} egress mechanism is catalyzed by a Na^+/Ca^{2+} antiporter which mediates the efflux of Ca^{2+} in exchange for the influx of Na^+. The inward Na^+ gradient mediated by the electroneutral Na^+/H^+ antiporter provides the driving force for Na^+/Ca^{2+} antiporter.

operates at its half-maximum velocity activated by the cytoplasmic Na^+ concentration. As a result, in physiological conditions, the two opposite Ca^{2+} transport routes would operate at approximately the same rate (Carafoli, 1979; Crompton et al., 1978). The release leg of the cycle (Na^+/Ca^{2+} exchanger in heart mitochondria) operates at a relatively constant slow rate, whereas the electrophoretic uptake leg oscillates below and above the rate of the release exchanger, responding essentially to the fluctuations in the cytosolic Ca^{2+} (Carafoli, 1988).

1.2.5 Physiological and Pathological Significance of the Mitochondrial Ca^{2+} Transport System

The kinetic properties of the mitochondrial Ca^{2+} transport system and the further discovery of the Ca^{2+}-sensitive matrix dehydrogenases lead to a proposal that the role of the mitochondrial Ca^{2+} transport system is to regulate intramitochondrial Ca^{2+} and to relay changes in cytoplasmic Ca^{2+} into the mitochondrial matrix (Denton & McCormack, 1980). According to this theory, Ca^{2+} cycling provides a mechanism by which increases and decreases in the cytosolic free Ca^{2+} level can be translated into parallel changes in the concentration of Ca^{2+} within the mitochondria. This mechanism allows the activities of the Ca^{2+}-sensitive dehydrogenases, and possibly the overall rate of oxidative ATP synthesis, to be sensitive to changes in the cytosolic Ca^{2+} concentration. In this manner, hormones and other extracellular messengers which stimulate ATP-requiring processes, such as secretion or muscle contraction through increasing the cytoplasmic concentration of Ca^{2+}, also could increase intramitochondrial oxidative metabolism and hence promote the replenishment of ATP (Denton & McCormack, 1985), as shown in Figure 1.2.

Therefore, although the role of the mitochondrial Ca^{2+} transport system in the regulation of cytosolic Ca^{2+} under physiological conditions is probably minor, under pathological conditions of excessive Ca^{2+} influx into cells, e.g., after ischaemia-reperfusion in the heart, the mitochondrial Ca^{2+} influx route is activated and buffers the cytoplasmic

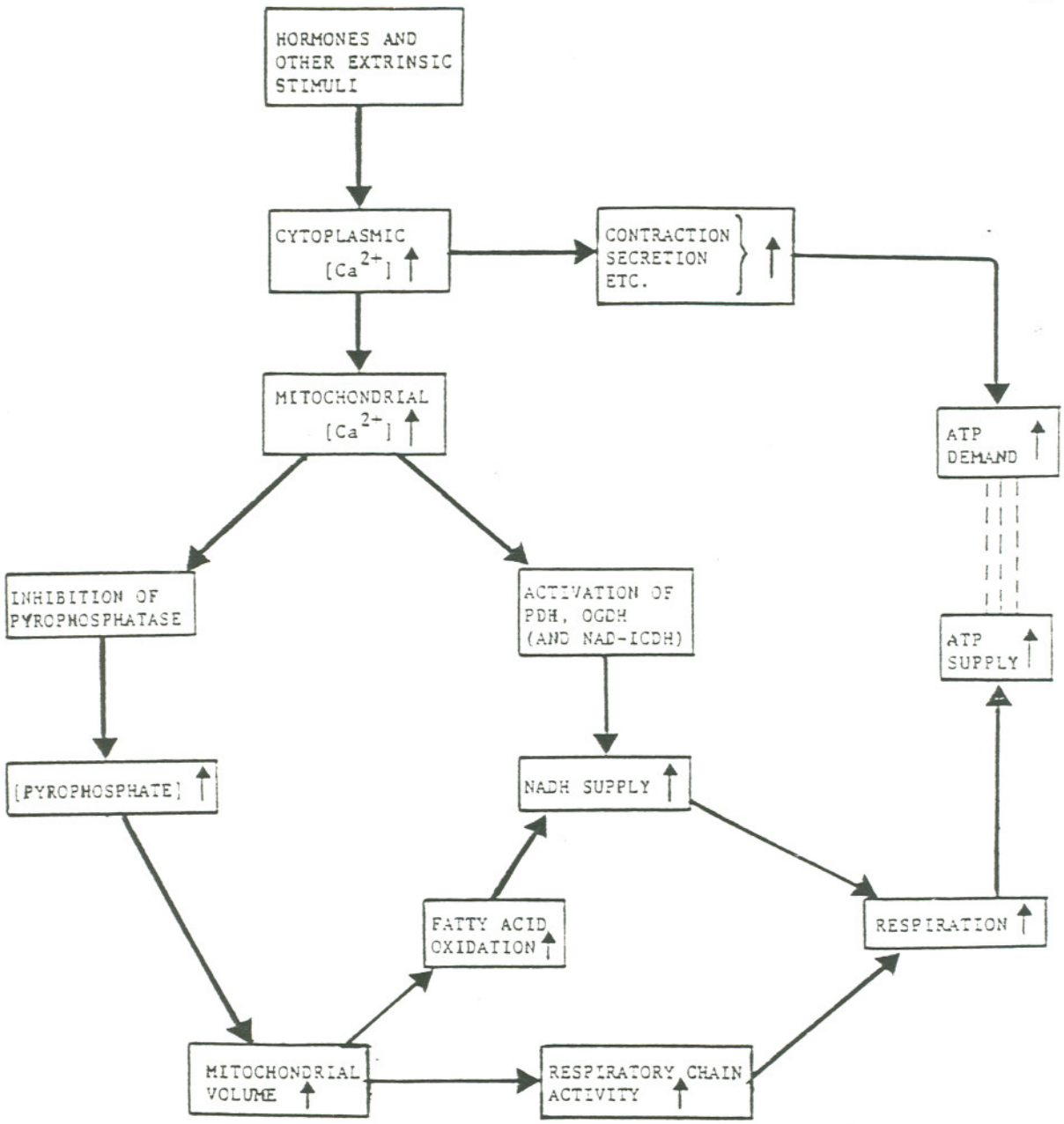

Fig 1.2 An integrated view of role of Ca^{2+} in stimulation of intramitochondrial metabolism, emphasizing its importance in increasing supply of ATP under conditions of increased energy demand.

Ca^{2+} by uptaking Ca^{2+} and storing it in Ca^{2+}-phosphate granular deposits (McCormack, 1987; Yamada, 1988). Thus, mitochondria act as a safeguard against cytosolic Ca^{2+} overload.

1.3 The Mitochondrial Na^+/Ca^{2+} Antiporter

1.3.1 The Nature of the Na^+/Ca^{2+} Antiporter

In the 1970s, Carafoli and co-workers (1974) discovered that Na^+ can stimulate profound and rapid calcium efflux in heart mitochondria in the presence of a uptake pathway blocker, ruthenium red, and this study was extended by Crompton et al. (1976, 1977, 1978), which identified a specific carrier that exchanges external Na^+ for intramitochondrial Ca^{2+} — a Na^+/Ca^{2+} antiporter. This mechanism is most prominent in mitochondria of excitable tissues, such as the brain, adrenal cortex, parotid gland, cardiac and skeletal muscle. The velocity of the Na^+-induced efflux of Ca^{2+} from mitochondria exhibits a sigmoid dependence on the $[Na^+]$, reaching a half-maximal velocity at 8 mM external Na^+, the range of the Na^+ concentration presumed to exist in most cytoplasm (Crompton et al., 1978). The maximum capacity of the efflux components of the system appears to be ~5-10 nmol of calcium released per minute per mg mitochondrial protein, ~2% of the maximum capacity of the uptake system (Coll et al., 1982; Nicholls, 1982).

Another key parameter for the Na^+/Ca^{2+} antiporter is the K_m for Ca^{2+}. Estimation of this parameter in isolated mitochondria was technically difficult and the reported values ranged from 2 to 13 μM free Ca^{2+} (Crompton et al., 1977; Hayat & Crompton, 1982; Coll et al., 1982). The exchange has been fit to a first-order Ca^{2+} dependence and a second- or third-order Na^+ dependence (Crompton et al., 1977; 1978). A tentative Ping-Pong mechanism has been proposed based on the kinetic studies (Crompton et al., 1977; Hayat & Crompton, 1982).

The binding of Na^+ and Ca^{2+} to transport sites is competitive (Hayat & Crompton, 1982). Li^+ can substitute for Na^+ with velocity about one third of that by sodium with K_m

of about 15 mM (Crompton et al., 1978). External K^+ is a strong activator of this mechanism (half-maximal activation ≈ 18 mM), but K^+ cannot substitute for Na^+ (Crompton et al., 1980). The cytosolic Na^+ concentration is reported to be between 5 to 10 mM (Lee et al., 1975) and matrix Ca^{2+} ranges from 0.1 to 0.8 μM (Moreno-Sanchez et al., 1988). Considering the physiological Na^+ and Ca^{2+} ion concentrations, K^+ activation, and higher temperature *in vivo*, the Na^+/Ca^{2+} antiporter is expected to be relatively active and has high turnover number, which is consistent with its role of regulating and maintaining the matrix Ca^{2+} level at physiological level.

1.3.2 Stoichiometry

Early in Na^+/Ca^{2+} antiporter research, kinetic measurements suggested that there were three independent binding sites for Na^+, which led to the hypothesis of $3Na^+/1Ca^{2+}$ exchange (Crompton et al., 1976, 1977). However, Affolter and Carafoli (1980) monitored the membrane potential in heart mitochondria did not alter measurably when Ca^{2+}/nNa^+ antiport was induced on respiring, ruthenium red-treated mitochondria. This result demonstrated that this carrier was electroneutral, a $2Na^+/1Ca^{2+}$ exchange. The electroneutrality of Na^+/Ca^{2+} exchange was further supported by Brand's experiment, which showed that the equilibrium of $[Ca^{2+}]$ maintained by Na^+/Ca^{2+} antiporter was not disturbed by adding ionophore A23187 (which catalyses $Ca^{2+}/2H^+$ exchange), and this agreed with the hypothesis of the stoichiometry being $2Na^+$ to $1Ca^{2+}$ (Brand, 1985). However, recent studies by Baysal et al. (1994) questioned the electroneutrol stoichiometry of the Na^+/Ca^{2+} antiporter. They proposed that $\Delta\psi$ might contribute to the Na^+-dependent Ca^{2+} efflux; therefore, the Na^+/Ca^{2+} antiporter is electrogenic — a $3Na^+/1Ca^{2+}$ exchange.

1.3.3 Regulation and Modulation

1.3.3.1 Ca^{2+} Regulation

It was observed by Hayat and Crompton (1982) that the Na^+-induced efflux of Ca^{2+} catalyzed by the Na^+/Ca^{2+} antiporter of cardiac mitochondria is strongly inhibited by extramitochondrial Ca^{2+}. The V_{max} for this process is decreased about 70% by cytoplasmic Ca^{2+}, which can bind to external sites cooperatively at half saturated concentration of 0.7-0.8 μM free Ca^{2+} (Hayat & Crompton, 1982). There is a competitive relation between the binding of external Na^+ and external Ca^{2+} to substrate-binding sites (those sites involved in cation translocation), whereas at low concentration (<4 μM) extramitochondrial Ca^{2+} is a partial non-competitive inhibitor with respect to external Na^+. The evidence outlined above suggests that the Na^+/Ca^{2+} antiporter may contain regulatory sites on the external face of the inner membrane, which render the carrier highly sensitive to changes in extramitochondrial free Ca^{2+} within the physiological range (Hayat & Crompton, 1982).

1.3.3.2 Mg^{2+} and Adenine Nucleotide Regulation

Hayat and Crompton (1987) also found that at 2.5 mM external free Mg^{2+} — the expected cytosolic Mg^{2+} level — the Na^+/Ca^{2+} antiporter activity could be effectively blocked; however, this inhibitory effect was largely abolished by ATP or ADP with external [Mg^{2+}] maintained constant. The non-competitive inhibition by Mg^{2+} on the Na^+/Ca^{2+} antiporter indicated that Mg^{2+} may substitute for Ca^{2+} in interacting with the regulatory sites on cytosolic surface of the mitochondrial Na^+/Ca^{2+} antiporter (Hayat & Crompton, 1987). Therefore, the regulatory sites are effective in controlling the Na^+/Ca^{2+} antiporter at physiological concentrations of adenine nucleotides, Mg^{2+}, intro- and extra-mitochondrial free Ca^{2+}. High matrix Ca^{2+} activates the ATP production process and increases the phosphorylation potential. The increased ATP can release Na^+/Ca^{2+} antiporter from Mg^{2+} inhibition and decrease matrix Ca^{2+}, hence the ATP production.

1.3.3.3 β-Adrenergic and Glucagon Regulation

The activation of the Na^+/Ca^{2+} antiporter by β-adrenergic agonists and glucagon in isolated mitochondria has been reported by Goldstone and Crompton (1982, 1983). Their data indicated that cyclic AMP may mediate the activation caused by glucagon, but cyclic AMP is not linked to the β-adrenergic induced mitochondrial Na^+/Ca^{2+} antiporter activation.

1.3.3.4 pH Regulation

The effect of matrix pH on the activity of the mitochondrial Na^+/Ca^{2+} antiporter has been studied using the fluorescence of SNARF-1 to monitor matrix pH and Na^+-dependent efflux of accumulated Ca^{2+} to follow antiport activity. The optimum rate of Na^+/Ca^{2+} antiporter is obtained at matrix pH 7.5 to 7.6, and changes in antiport activity do not correlate with changes in components of the driving force of the reaction (i.e., $\Delta\psi$, ΔpH, or the steady state Na^+ gradient) (Baysal, 1991). Hence, the Na^+/Ca^{2+} antiporter of heart mitochondria is very sensitive to matrix $[H^+]$, and changes in matrix pH may contribute to the regulation of matrix Ca^{2+} levels.

1.3.3.5 Benzothiazepines

Certain benzothiazepines, particularly diltiazem, can effectively inhibit the Na^+/Ca^{2+} antiporter with similar potency in isolated mitochondria (Vaghy et al., 1982; Martib & Schwartz, 1983) and in purified carriers (Li et al., 1992). Although these drugs are well known for their Ca^{2+}-channel blocking activity, their effects on mitochondrial Na^+/Ca^{2+} antiporter may also explain some of the important function achieved by these drugs. The antagonism of exchange activity by diltiazem is stereospecific, with D-cis-diltiazem being much more potent and selective to mitochondrial Na^+/Ca^{2+} antiporter, while the L-cis-isomer is more potent to Ca^{2+}-channel (Cox & Martib, 1993b). The stereospecificity of the action of diltiazem suggests a specific interaction of this compound with the exchanger

molecule. Clonazepam has the same potency as D-cis-diltiazem to mitochondrial Na^+/Ca^{2+} antiporter, but the clonazepam has no affect on the voltage-dependent Ca^{2+} channel of cardiac muscle (Martib & Schwartz, 1983). CGP-37157 selectively inhibits the activity of mitochondrial Na^+/Ca^{2+} exchange in a dose dependent manner (IC_{50}=0.36 μM) without affecting the Ca^{2+}-channel, the Na^+/Ca^{2+} exchanger of the cardiac sarcolemma, or the Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum (Cox & Matlib, 1993b).

1.3.3.6 Lanthanide Series and Other Cations

In early studies, the lanthanide series was employed to inhibite the mitochondrial Na^+/Ca^{2+} antiporter (Crompton et al., 1977; 1979), and these metal cations provide very good tools to study the Na^+/Ca^{2+} antiporter. Ba^{2+} can effectively inhibit the Na^+-dependent Ca^{2+} release non-competitively in heart mitochondria, with respect to extramitochondrial Na^+ (Lukacs & Fonyo, 1986). It was thought that Ba^{2+} acts at the regulatory binding site of the Na^+/Ca^{2+} antiporter at the cytoplasmic surface of the inner mitochondrial membrane, the same site where extramitochondrial Ca^{2+} regulates antiporter activity.

1.3.4 Distribution of the Na^+/Ca^{2+} Antiporter

The mitochondrial Ca^{2+}-transport system is found to exit in mammalian tissues in many species. The Na^+/Ca^{2+} exchange is predominant in tissues, like the heart, brain, adrenal cortex, parotid gland, and skeletal muscle (Crompton et al., 1978). There also appears to be a Na^+-independent egress process which remains poorly characterized, but is thought perhaps to catalyze direct Ca^{2+}/H^+ exchange (Crompton, 1985; Hansford, 1985; Gunter & Pfeiffer, 1990). For a long time the latter pathway was advocated as the major egress pathway in non-excitable tissues such as liver and kidney. However, recently more careful experimentation using more physiological mitochondrial Ca^{2+} loads suggests that Na^+/Ca^{2+} exchange is also predominates in these tissues (McCormack, 1985; 1988). The

observation of the Na^+-independent pathway may be an artifactor of work on isolated mitochondria and their having supraphysiological Ca^{2+} loads (McCormack et al., 1992).

1.4 The Physiological Significance of the Na^+/Ca^{2+} Antiporter

The role of the Na^+/Ca^{2+} antiporter in cellular function of heart mitochondria is not yet clear. Early theories suggested that Ca^{2+} release from heart mitochondria mediated by Na^+/Ca^{2+} exchange may play a role in regulation of cytosolic free Ca^{2+} (Mela, 1977). However, the kinetic characteristics of mitochondrial Na^+/Ca^{2+} exchange under physiological conditions are inconsistent with a role in the beat-to-beat regulation of the cytosolic free Ca^{2+} in the heart (Gunter & Pfieffer, 1990). Since the major physiological role of mitochondrial Ca^{2+} transport system is to regulate its own Ca^{2+} level, the role of the Na^+/Ca^{2+} antiporter — an element of the transport system — probably focuses on regulation of matrix Ca^{2+} activity.

Cox and Matlib (1993a) investigated the effects of stimulation and inhibition of Na^+/Ca^{2+} antiporter on the matrix free Ca^{2+} concentration in isolated heart mitochondria and the consequences of these changes on the rate of NADH production via Krebs cycle turnover and the oxidative phosphorylation rate. Activation of the mitochondrial Na^+/Ca^{2+} antiporter by extramitochondrial Na^+ leads to Ca^{2+} efflux and the consequent decrease in matrix free $[Ca^{2+}]$. Inhibitors of the mitochondrial Na^+/Ca^{2+} exchange system, like the CGP-37157, clonazepam, and d-cis diltiazem, cause a dose-dependent increase in matrix free $[Ca^{2+}]$ until the exchanger is completely blocked (Cox & Matlib, 1993a). Stimulation and inhibition of Na^+/Ca^{2+} antiporter activity can decrease and increase, respectively, the rate of Krebs cycle turnover via alteration in matrix free $[Ca^{2+}]$. Therefore, the Na^+/Ca^{2+} antiporter is a major regulatory site for mitochondrial Ca^{2+} cycle. The role of the mitochondrial Na^+/Ca^{2+} exchanger is to maintain matrix free Ca^{2+} level within a range that is capable of regulating the activity of the Ca^{2+}-sensitive dehydrogenases. The alterations in matrix free $[Ca^{2+}]$ induced by changes in mitochondrial Na^+/Ca^{2+} antiporter activity are

translated into changes in the rate of NADH production and the overall rate of oxidative phosphorylation.

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

Despite the increasing recognition of the importance of the mitochondrial Na^+/Ca^{2+} antiporter in regulation of matrix Ca^{2+}, which in turn controls mitochondrial enzymes to meet the cell demands for ATP, very little is as yet known about the molecular nature of this carrier. Many groups (McCormack; Matlib; unpublished work) including our lab (Garlid) are trying to purify and explore the molecular nature of the Na^+/Ca^{2+} antiporter. Our lab first reported partial purification of reconstitutively active Na^+/Ca^{2+} exchange as a 110-kDa protein from beef heart mitochondria (Li et al., 1992).

Our lab first observed Na^+/Ca^{2+} exchange in proteoliposomes reconstituted with total extract of beef heart submitochondrial particles during previous studies on the Na^+/H^+ antiporter (Garlid et al., 1991). We undertook to purify this activity by reconstituting fractions from DEAE-cellulose chromatography columns. Na^+/Ca^{2+} exchange was characterized with the Na^+-selective fluorescent probe, SBFI, and the Ca^{2+}-selective fluorescent probe, Fura-2. Reconstituted Na^+/Ca^{2+} exchange was inhibited by diltiazem and TPP^+, as observed in intact mitochondria. A new finding was that this protein can catalyze Na^+/Li^+ exchange in the absence of Ca^{2+}. Polyclonal antibodies were raised against the 110-kDa protein fraction that had active Na^+/Ca^{2+} exchange activity, and the 110-kDa protein specific antibody inhibited Na^+/Ca^{2+} exchange (Li et al., 1992).

Screening a λgt11 cDNA expression library using 110-kDa protein specific polyclonal antibodies as probe, the selected positive clones proved to be mitochondrial nicotinamide nucleotide transhydrogenases (TH). This result led us to realize that the 110-kDa protein band contained TH contamination in addition to the putative Na^+/Ca^{2+} antiporter, and that TH occupied a significant proportion in DEAE-cellulose purified 110-kDa protein band. Therefore, the 110-kDa band specific antibodies were mixtures of TH

antibodies and Na^+/Ca^{2+} antiporter antibodies. For separation of the two proteins at 110-kDa and identification of the Na^+/Ca^{2+} antiporter, a partial TH antigen was expressed in *E. coli*, and TH antibodies against the expressed TH antigen were immunopurified. The Rotofor-Cell technique separated the two proteins at 110-kDa with different pI. To remove TH contamination in the 110-kDa protein fraction preparatively, a TH antibody immobilized chromatography column was used to absorb TH protein in the mixture 110-kDa protein fraction, and the resultant flow-through fraction contained mainly the putative Na^+/Ca^{2+} antiporter with little TH contamination. The antibodies specific to this negatively purified 110-kDa protein showed inhibition of Na^+/Ca^{2+} transport, while the TH antibodies did not. This thesis mainly describes discovering the TH contamination, identifying the existence of the Na^+/Ca^{2+} antiporter in the 110-kDa protein mixture and separating these two proteins.

Chapter 2
Materials and Methods

2.1 Materials

The PQE expression and purification system and the plasmid DNA Mini-prep kit were purchased from QIAGEN Inc. The bovine heart λgt 11 cDNA library was obtained from Clontech. Sequenase 2.0 and reagents for sequencing were purchased from USB, and the Geneclean reagents from BIO101, La Jolla, CA. CIP and T4 DNA ligase were from New England Biolab. Restriction enzymes Pst I and Bst EII were supplied by GIBCO BRL. AminoLink coupling gel was bought from Pierce, and detergent adsorber gel was from Boehringer Mannhem. Rotofor-Cell, Bio-Lyte Ampholyte, and electrophoresis reagents were obtained from Bio-Rad. ECL Western blot reagents were purchased from Amersham. Other chemicals and reagents were supplied by Sigma.

2.2 Partial Purification of Mitochondrial Na^+/Ca^{2+} Antiporter

Preparation of submitochondrial particles (SMP) followed the previously described procedure (Garlid et al., 1991). To solubilize membrane proteins, 200 mg SMPs were solubilized in 25 mM TEA-TES, 0.1 mM TEA-EGTA, 1 mM DTT, 20% glycerol, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, and 3% Triton X-100, pH 7.3, at a concentration of 4 mg protein/ml. After incubation for 1 h, the supernatant which contained ~180 mg protein extract of 3-4 mg/ml was collected by centrifugation at 130,000 *g* for 30 min. Two DEAE-cellulose chromatographies were used sequentially to purify the Na^+/Ca^{2+} exchange active fraction as previously described (Li et al., 1992).

2.3 Reconstitution and Fluorescence Measurements

Reconstitution and antibody inhibition generally followed the previous protocols (Li et al., 1992), except the phospholipid was mixture of 90% Asolectin with 10% Cardiolipin and a 2.7 ml Bio-Beads column was used to remove detergent. The antibody titer was adjusted by Grid-Blot method described below.

The fluorescence emission intensities of SBFI and Fura-2 are enhanced in the presence of Na^+ and Ca^{2+}, respectively. Probe-loaded proteoliposome fluorescence was measured and calibrated generally as previously described (Garlid et al., 1991; Jezek et al., 1990). Free [Ca^{2+}] was estimated using the Chelator program (Schoenmakers et al., 1992).

2.4 SDS/PAGE, Western Blot and Immunopurification Antibodies

SDS-PAGE was performed according to Laemmli and Favre (1973). Transblotting of proteins from SDS/polyacrylamide gels on nitrocellulose membrane and Western blot analyses were performed follow protocols of Blake et al. (1984) with second antibody conjugated with alkaline phosphatase or horseradish peroxidase (ECL Western blot, Amersham Life Science). The 110-kDa band specific antibodies were immunopurified from antiserum raised against the active 110-kDa protein fraction as described (Li et al., 1992).

2.5 Immuno-screening the Bovine Heart λgt11 cDNA Library

Standard techniques (Sambrook et al., 1989) were used for screening, subcloning and sequencing. A commercial λgt11 bovine cardiac expression cDNA library (5'-stretch, Clontech) was probed with the 110-kDa protein band specific polycolonal antibodies (designated as 110 antibody-1). A positive clone was selected from 2×10^6 plaques, and the insert of 2.3 kb was subcloned into the EcoR I site of plasmid pUC19 vector. This clone was sequenced using the dideoxy-chain termination method (Sanger and Coulson,

1975). As described in result, this clone, designated as pTH13, is actually a partial cDNA clone of mitochondrial nicotinamide nucleotide transhydrogenases (TH).

2.6 Expression of Transhydrogenase in *E. coli*

2.6.1 Expression Vector Plasmid and Bacterial Strain

The pQE plasmids (QIAGEN Inc.) belong to the pDS family of plasmids and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS. They contain the bacteriophage T5 promoter and two *lac* operator sequences. The control region of pQE vectors is shown in Figure 2.1. The pQE30, 31 and 32 vectors are in three reading frames belonging to the same construct, which places a 6×His tag at the N-terminus of the expressed protein. The genotype of host bacterial strain M15 is: *Nal*^{s} *Str*^{s} *rif*^{s} *lac*$^{-}$ *ara*$^{-}$ *gal*$^{-}$ *mtl*$^{-}$ *F*$^{-}$ *recA*$^{+}$ *uvr*$^{+}$. M15 contains another plasmid pREP4, which carries the *lacI* gene encoding the *lac* repressor. The restriction map of the pQE vector and pREP4 are illustrated in Figures 2.2 and 2.3. Expression from pQE vectors is induced by the addition of IPTG, which inactivates the repressor and clears the promoter.

2.6.2 Preparation of the Subcloning Insert Fragment

A single bacterial XL-1 blue colony (containing pTH13) was inoculated and grown in 5 ml LB medium with 50 μg/ml of ampicillin and cultured overnight at 37°C with shaking. The plasmid pTH13 DNA was extracted using silicon chromatography QIAgen Miniprep kit. The pTH13 plasmid DNA was digested with enzyme Pst I at 37°C for 2 h, then the cleaved DNA was fractionated on 1% agarose gel. The 888 bp fragment of insert was excised from the gel and purified by the Geneclean procedure (BIO101, La Jolla, CA).

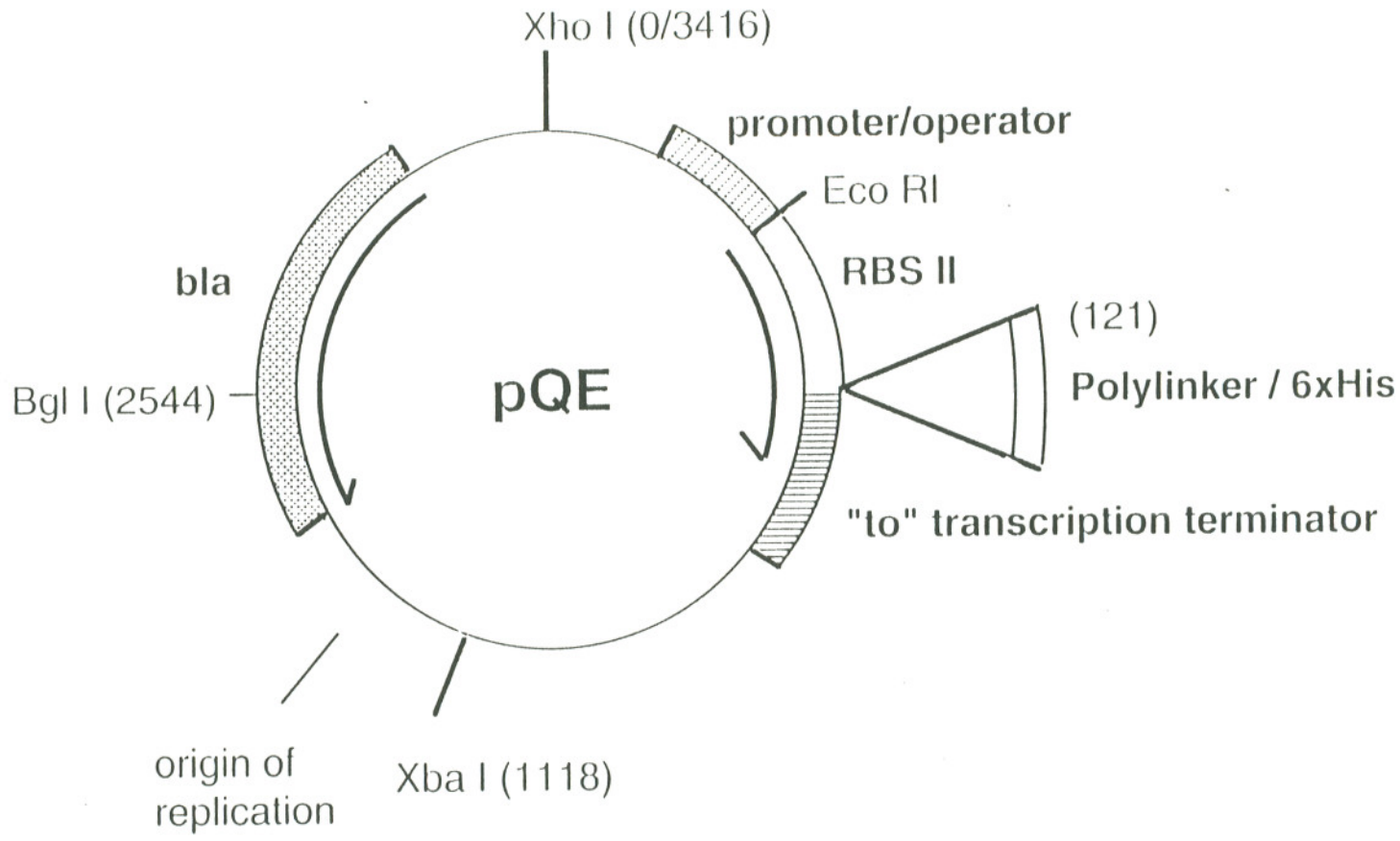

Fig 2.1 Control region of QIAexpress pQE-vectors

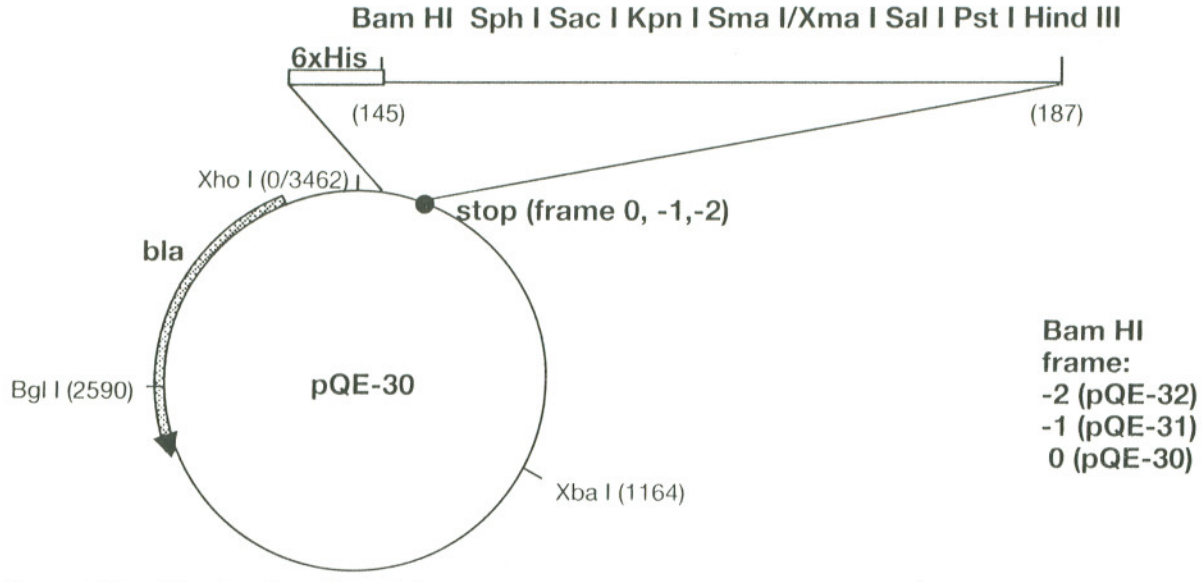

Figure 10a: Cloning into Type IV vectors.

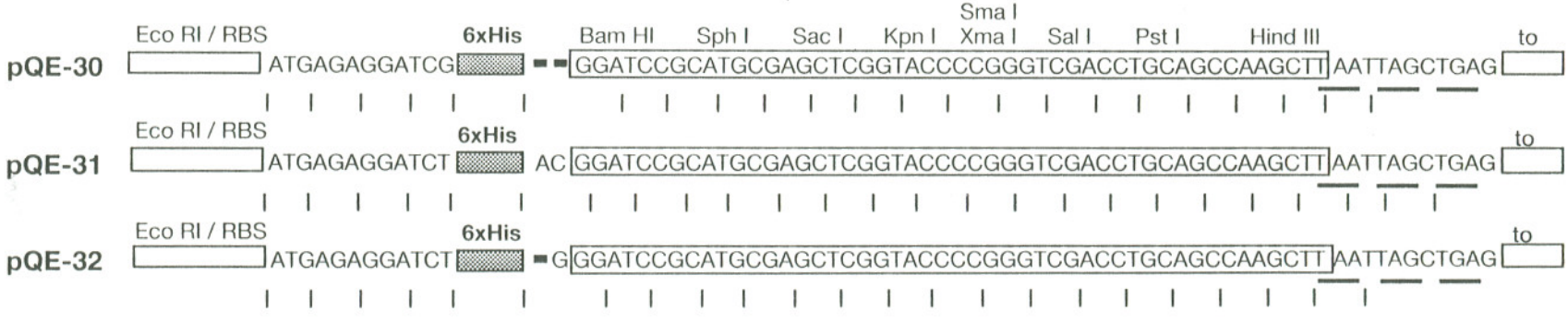

Fig 2.2 Restriction map of pQE 30, 31 and 32 vectors

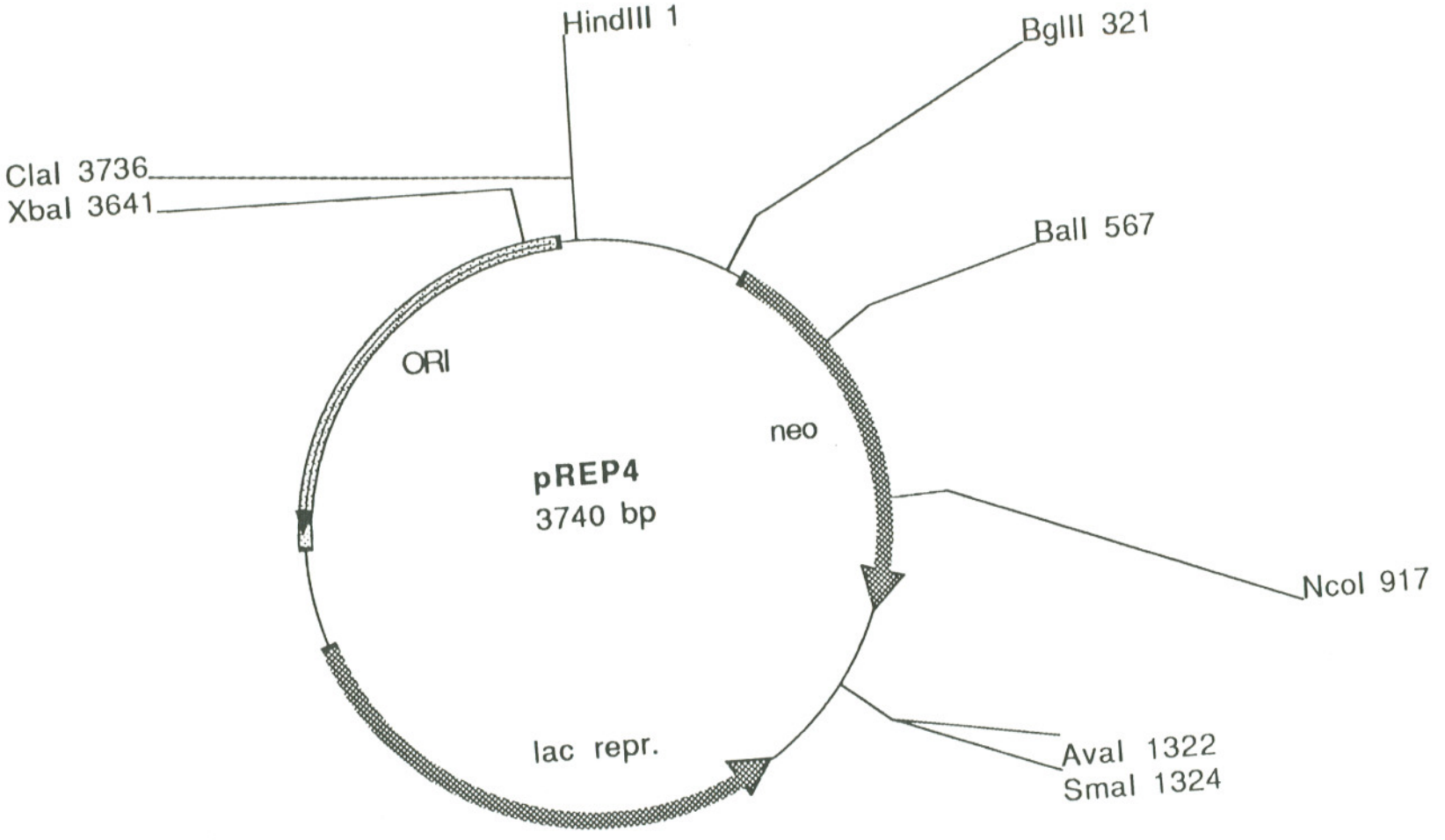

Fig 2.3 Restriction map of pREP-4

2.6.3 Construction of Expression Plasmids

The 888 bp Pst I fragment corresponding to nucleotides 572-1460 of the bovine heart transhydrogenase cDNA (Yamaguchi & Hatefi, 1988) was directly subcloned into the Pst I site of the polylinker, downstream of the T5 promoter of the expression vectors pQE 30, 31 and 32, respectively.

The pQE vectors were linearized with digestion of Pst I, then the 5'-phosphate groups were dephosphorylated with calf intestinal alkaline phosphatase (CIP) by incubation at 50°C for 1h. The cleaved and dephosphorylated vector DNA was subjected to 1% agarose gel, and the linear vector DNA of 3.46 kb was excised from the gel and purified by the Geneclean procedure. The linearized pQE vectors were ligated to the 888 bp insert catalyzed by bacteriophage T4 DNA ligase at 16°C for 12 h. The ligation mixture was transformed into Ca^{2+}-treated M15 [pREP4] competent cells. Preparation of M15 [pREP4] competent cells and transformation followed the procedure recommended by QIAGEN.

2.6.4 Identification of Recombinant Transformants

The pQE expression plasmids do not provide color selection of plasmids that contain inserts after ligation and transformation, so we chose enzyme mapping method to identify the transformants that contain inserts, which are extraction of the transformant DNA and digestion of the circular plasmid DNA by restriction enzyme Pst I and Bst EII.

A single transformant colony was inoculated in 3 ml LB medium containing 25 μg/ml kanamycin and 100 μg/ml ampilcillin at 37°C overnight. Plasmid DNA was extracted by boiling method (Sambrook et al., 1989), then cut by Pst I and Bst EII, simultaneously, at 37°C for 2 h. The cleaved DNA was fractionated on 1% agarose gel.

2.6.5 Bacterial Expression of the Recombinant Protein

A single colony of transformant, which was confirmed by enzyme mapping that the recombinant pQE plasmid did contain the insert, was inoculated into LB medium containing 25 μg/ml kanamycin and 100 μg/ml ampilcillin 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. Then IPTG was added to a final concentration of 1-2 mM to induce the expression of the recombinant protein, and incubation was continued for a further 4 h. A control culture containing the pQE-16 transformant, which expresses dihydrofolate reductase (DHFR) at MW of 26-kDa, was grown at the same time. The cells were harvested by centrifugation, and either were used immediately for preparation of SDS-PAGE or were frozen for purification.

2.6.6 Purification of Recombinant Protein

The expressed proteins that contain six consecutive histidine residues — the 6His affinity tag — at their N-terminus can be affinity purified by Ni-NTA resin with high affinity ($K_d = 10^{-13}$, pH 8.0). The interaction between 6xHis tag and Ni-NTA resin is shown in Figure 2.4.

Cells were resuspended in buffer A (6 M GuHCl, 0.1 M Na-phosphote, 0.01 M Tris/HCl, pH 8.0) at 5 ml per gram wet weight, then stirred for 1 h at room temperature. Cell lysate was centrifuged at 10,000 × *g* for 15 min at 4°C, then Ni-NTA resin was added to the supernant (8 ml of 50% slurry of Ni-NTA resin for 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 buffer A, 5 column volumes of buffer B (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 8.0) and buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 6.3) until the A_{280} is < 0.01. The recombinant protein was eluted with 10-20 ml buffer D (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH 5.9), followed by 10-20 ml buffer E (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl, pH

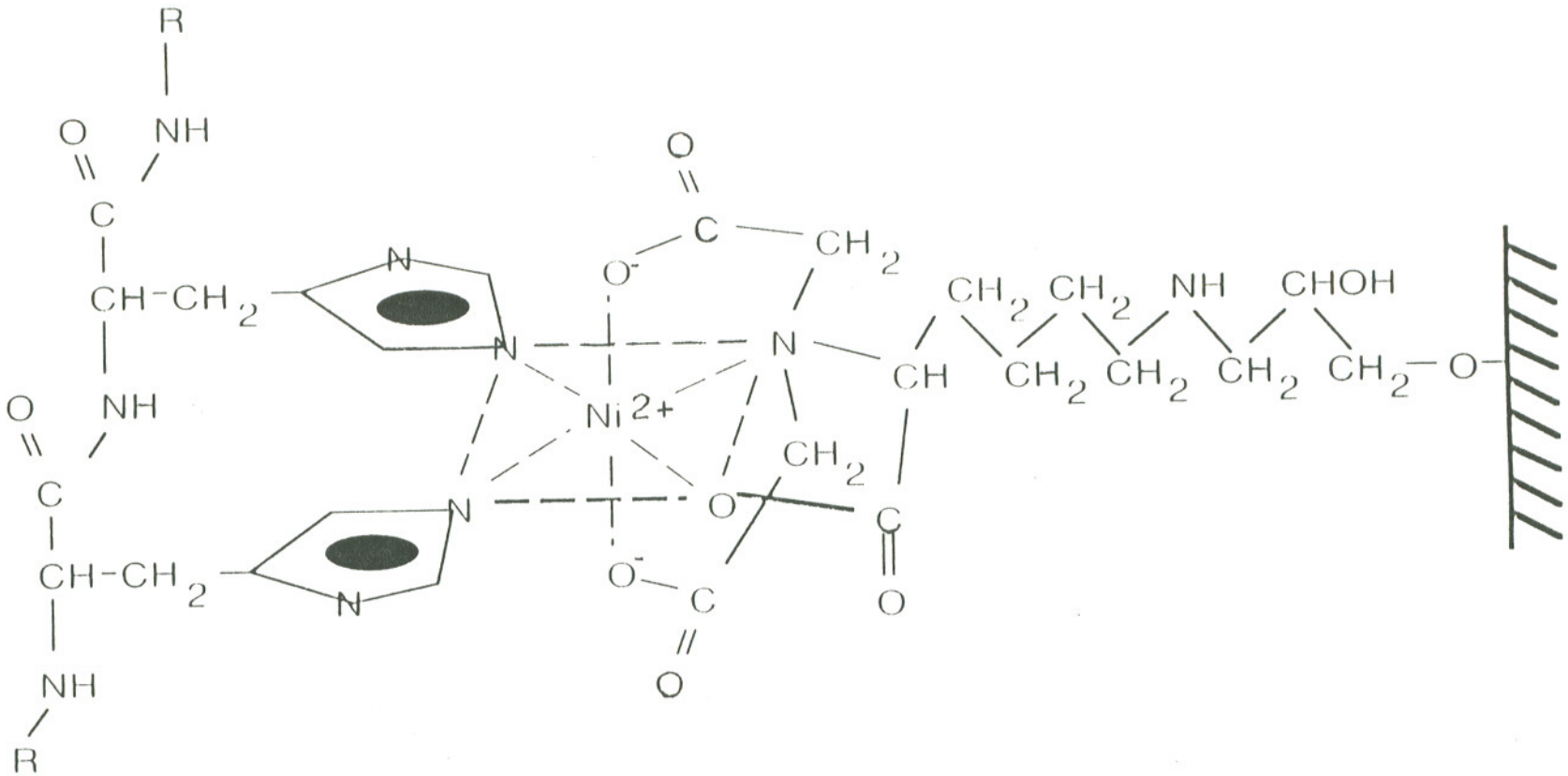

Fig 2.4 Interaction between 6xHis tag and Ni-NTA resin. The NTA ligand has four chelating sites which can interact with metal ions. Thus NTA occupies four of the six ligand binding sites in the coordination sphere of Ni^{2+} ion, leaving two sites free to interact with the 6xHis tag.

4.5), and 20 ml buffer F (6 M GuHCl, 0.2 M acetic acid). 3 ml fractions from each elution were collected and analyzed by 10% SDS-PAGE and by Western blot with the 110 antibody-1.

2.7 Rotofor-Cell Separation of TH Protein and Na^+/Ca^{2+} Antiporter

The Rotofor-Cell (Bio-Rad) is a preparative scale, free solution, isoelectric focusing apparatus for purifying proteins. The focusing chamber was filled with 50 ml of Na^+/Ca^{2+} exchange active DEAE-cellulose column purified 110-kDa protein fraction, which contained 0.5 mg of 110-kDa protein, in the buffer of 5 mM TEA-TES, 1 mM DTT, 0.1 mM EGTA, 1% Triton X-100, 20% glycerol, 1% (W/V) Bio-Lyte 6/8 Ampholyte, and 1% Bio-Lyte 8/10 Ampholyte. The sample was focused in 4 h with 12 W at rotating of 1 rpm. The 20 fractions held by 20 discrete compartments in the focusing chamber were rapidly collected without mixing. Each fraction of about 2.5 ml was measured for pH and subjected to 7.5% SDS-PAGE, then analyzed by Western blot with 110 antibody-1.

2.8 Purification of the Na^+/Ca^{2+} Antiporter by Immuno-affinity Chromatography

Applying affinity chromatography to purity the Na^+/Ca^{2+} antiporter is based on having high quality TH antigen expressed in bacteria and TH antibodies immunopurified against the expressed TH antigen. TH antibodies were covalently coupled to the insoluble matrix (agarose) of AminoLink™ gel (Pierce) by activating the agarose to yield aldehydes that react with primary amine groups of proteins to form Schiff bases. The solid phase TH antibodies specifically bind to TH proteins in a mixture fraction; therefore, TH protein can be removed from the 110-kDa mixture protein fraction, achieving our goal of separating the Na^+/Ca^{2+} antiporter.

2.8.1 Immobilization of TH Antibodies to AminoLink Column

Antibodies specific to the expressed TH antigen were purified from antiserum raised against the active 110-kDa protein fraction as previously described (Li et al., 1992). The TH antibodies were concentrated by Centricon-50 (Amicon), and tris-glycine buffer was changed to PBS coupling buffer (0.1 M Na-phosphate, 0.05% Na-Azide, pH 7.0) by repeated concentration, followed by redilution of the concentrated sample with PBS. 2 ml of TH antibody solution with a concentration of 2 mg/ml were applied to a PBS-equilibrated 1 ml AminoLink™ gel column (Pierce) in the presence of 0.1 ml of 1.0 M $NaCNBH_3$ as reductant. The gel was mixed with the antibody solution at room temperature for 2 h, then left to stand at 4°C for an additional 4 h. The effluent was collected, and the column was washed with 6 ml PBS coupling buffer. The coupling efficiency was determined by comparing the antibody amount of the original solution to that in the effluent using the Amido Black protein estimation method (Kaplan & Pedersen, 1985).

The column was equilibrated with 4 ml of 1.0 M Tris-HCl, pH 7.4, quenching buffer. The sites on the gel uncoupled to antibodies were blocked by 2 ml of quenching buffer and 0.1 ml of 1.0 M $NaCNBH_3$ for 30 min, mixing at room temperature. The gel was washed with 4 × 5 ml of 1.0 M NaCl wash solution, then stored at 4°C in 0.05% Na-Azide storage solution.

2.8.2 Concentration of 110-kDa Protein Fraction

The DEAE cellulose column purified 110-kDa protein fractions, which were Na^+/Ca^{2+} exchange active, were concentrated by an ultrafiltration cell (Amicon) with a filter membrane of 10,000 MW cut-off. Under 25 psi nitrogen pressure, a 95 ml total protein sample containing 1% Triton X-100 was concentrated to 5 ml at 4°C for 10 h with continuously stirring. Then the sample was rediluted in 50 ml PBS buffer containing 0.1 mM EGTA, 1 mM DTT, and 10 μg/ml leupeptin, pH 7.3, and concentrated to 5 ml.

2.8.3 Removing Extra Triton from the Concentrated 110-kDa Protein Sample

Triton X-100 concentration in the concentrated 110-kDa protein sample was measured by fluorescence with the excitation set at 280 nm (slit width 0.5 nm) and the emission set at 306 nm (slit width 4 nm). As described in the results, the 5 ml concentrated sample contained 12.6% Triton X-100. To keep the detergent concentration at about 1% in our protein sample, detergent absorber gel (Boehringer Mannheim) was used to remove extra Triton X-100. The detergent absorber gel (3.7 g), which was pre-equilibrated with PBS buffer, was incubated with the protein solution in a column for 5 min at room temperature, then the protein solution was recovered by collecting the flow-through. Protein recovery was determined by Amido Black, and the Triton X-100 concentration was monitored by fluorescence.

2.8.4 Immunopurification of the Na^+/Ca^{2+} Antiporter

The 5 ml 110-kDa protein sample, which had been treated by detergent absorber gel, was applied onto the TH antibodies immobilized AminoLink column and incubated at 4°C overnight with slow shaking. The flow-through was collected, and the column was washed with 20 ml PBS buffer containing 0.5% Triton X-100. The column-bound proteins were eluted by 3.5 ml of 0.1 M glycine, 0.5% Triton X-100, pH 2.8. 0.8 ml fractions of elution were collected, and each 0.8 ml fraction was neutralized by the addition of 40 ml of 1 M Tris-HCl, pH 9.5. The column was then neutralized and regenerated by washing with 10 column volumes of PBS containing 0.5% Triton X-100 and stored in 0.05% Na-Azide, and 0.5% Triton X-100 at 4°C for the next use. Protein samples at each step were analyzed by 7.5% SDS-PAGE and checked by Western Blot with antibodies against the 110-kDa protein (110 antibody-1) and antibodies against the expressed TH (TH antibody), respectively.

New antibodies immunopurified to the 110-kDa protein of the flow-through fraction from the AminoLink column were designated as 110 antibody-2, and the specificity of 110-kDa antibody-2 was checked by Western blot.

2.9 Grid-Blot Estimation of Antibody Titer

The surf-blot (Idea Scientific Co., MN) is a device that clamps parallel liquid channels on the surface of a blot. Grid-blot is a modified Western-blot method that blots antigens to form parallel strips on one big membrane using the surf-blot device; first antibodies are then bound to the membrane with the antibody strips vertical to the antigen strips. The grids where antigens and antibodies crisscross show signals if the antibody specifically recognizes the antigen. Both antigen and antibody can be diluted to stepped concentrations in one experiment; therefore, differences in signal intensities of different antibodies to one kind of antigen can be used as indicators to estimate antibody titers. In reverse, this method can also be used to compare concentrations of different antigens.

A wet 14 × 14 cm nitrocellulose membrane was clamped into the surf-blot sandwich; then each aliquot of 200 μl antigen sample was added into channels and incubated at room temperature for 1 h with gentle rocking. The membrane was removed from the surf-blot sandwich, rinsed with Tris-buffered saline, and blocked with 5% non-fat milk. The membrane with the antigen blotted side facing up to the channels was clamped onto the cleaned surf-blot apparatus again, with the channels vertical to the antigen strips. 200 μl antibody aliquots were added to each slot and incubated for 1 h. Then the membrane was rinsed, bound to second-antibody, and immuno-stained following the ECL Western blot procedure. Both antigens and first antibodies were diluted in stepped-decrease concentrations.

Chapter 3

Results

3.1 Separation Strategy Rationales

The difficulties in separating Na^+/Ca^{2+} antiporter from TH protein reside in the following similarities between the two proteins: (i) both proteins have a similar molecular weight; (ii) both are highly hydrophobic integral membrane proteins which may form micelle complexes together with detergent during the purification process; (iii) both are basic proteins in their denatured form (see Rotofor results); (iv) the polyclonal antibodies, as a means to follow purification, were raised against 110-kDa protein mixture containing both proteins — in another words, the antibodies are also mixtures; (v) functional assay, though considered an alternative means to follow purification, is actually not very conclusive in that some of the contaminant never can be ruled out completely. In addition, it is hard to reach a conclusion from negative results in functional assays which can always be considered as a failure in technique.

The separation was started with many general techniques used in protein purification such as different ion-exchange column chromatography (DEAE, phosphocellulose and hydroxylapatite), with different pH including pH gradients, different buffers (TES, HEPEs, Tris), different salts (TEA, Na^+, and K^+), different gradients (stepwise and continuous), and different detergents (CHAPS, sodium deoxycholate and Triton X-100). All these methods turned out to be ineffective in separating the two 110-kDa proteins.

More specific technologies were then applied and considered. Negative purification of Na^+/Ca^{2+} antiporter by NADH immobilized column chromatography was first considered because NADH is a specific ligand of TH. TH catalyzes hydride ion transfer between NAD(H) and NADP(H) coupled to proton translocation across the mitochondrial

inner membrane. As adenine nucleotides are also putative ligands for Na^+/Ca^{2+} antiporter (Hayat & Crompton, 1987), the Na^+/Ca^{2+} antiporter can bind to the nucleotide moiety of NADH. The pilot experimental result was consistent with this concern showing no separation.

Proteinase digestion was also considered. Although the integrated TH protein and Na^+/Ca^{2+} antiporter are 110-kDa in size, the degraded peptides of these two proteins after proteinase partial digestion should be different in size. The SDS-PAGE separated peptides can be transblotted onto NC paper and probed with antibodies. However, this approach can not be successful unless there is either the antibody specific to TH or the antibody specific to Na^+/Ca^{2+} antiporter. Unfortunately, none of them except mixed antibodies was available.

Rotofor-Cell, a liquid phase iso-electric focusing technique, was also tried in the separation effort. It was proved to be efficient in identification of the existence of two proteins at 110-kDa (see below); the yield, however, was not ideal in either quantity or quality. Precipitation near pI, the presence of detergent, and micelle complex formation with multiple proteins probably accounted for this unsatisfactory result.

The key to reaching the goal of separating these two proteins is to obtain authentic purity in one of four factors: Na^+/Ca^{2+} antiporter protein, TH protein, the antibody specific to Na^+/Ca^{2+} antiporter protein, and the antibody specific to TH protein. We finally arrived at a solution to this difficult problem from the technology of molecular biology. By taking advantage of a published TH cDNA sequence, we chose a cDNA fragment coded for a very antigenic peptide fragment of TH protein and designed an expression system in *E. coli* to express the antigen with a histidine-tag. The expressed product that could be affinity purified with Ni-NTA column would be pure TH antigen without any contaminant from the Na^+/Ca^{2+} antiporter protein. The immunopurified TH-specific antibody could be used to build an affinity column to purify TH protein directly from the fraction containing the 110-kDa band. The followings are the results we obtained based on this strategy.

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

The methods we used for solubilization of mitochondrial membrane proteins and partial purification of 110-kDa Na^+/Ca^{2+} exchange active fraction generally based on the protocols previously described in our lab (Li et al., 1992), except for the use of 3% Triton X-100 instead of 1% Triton X-100 to extract membrane proteins. This change increased the detergent/protein (W/W) ratio from 10:4 to about 10:1, and the mitochondrial membrane protein solubilization yielded 40% more than the previous method. This modification did not change the purification profile. The fraction containing 110-kDa protein with high Na^+/Ca^{2+} exchange activity was still in the wash fraction of the DEAE-cellulose column that was eluted after flow-through and during washing of the column with 2 bed volume of loading buffer. The 70-kDa protein which was demonstrated to be mitochondrial hsp70 (Li et al., 1992) was co-purified with 110-kDa protein in the Na^+/Ca^{2+} exchange active fraction at a similar retention profile as described in Li et al. (1992); the heat shock protein at 70-kDa could be removed by alkaline extraction prior to purification.

3.3 Mixture of Antibodies Leading to a Wrong Clone in Immunoscreening

The antibodies against 110-kDa protein (110 antibody-1) from DEAE column chromatography were used as probe to screen a λgt11 bovine cardiac cDNA library. An isolated positive clone, designated as pTH13, contained an insert of 2.3 kb. The nucleotide sequence of pTH13 was confirmed to correspond to the beef heart mitochondrial nicotinamide nucleotide transhydrogenases (TH) cDNA of nucleotide 501 to 2794 (Yamaguchi & Hatefi, 1988) by checking the GeneBank. This result indicated that the 110-kDa band specific antibodies contained TH antibody, which probed out the TH clone from the library. Therefore, we deduced that the DEAE-cellulose column purified 110-kDa protein contained TH protein. Much of our previous data (Li et al.,

1992) demonstrated that Na^+/Ca^{2+} exchange activity corresponded to mitochondrial membrane protein at 110-kDa; therefore, we assumed that the DEAE-cellulose purified 110-kDa protein band contained at least two proteins, the putative Na^+/Ca^{2+} antiporter and TH, and that the TH protein occupied a significant portion in the mixture.

3.4 Expression of Antigenic Part of Mitochondrial Transhydrogenase in *E. coli*

3.4.1 Construction of the Expression Plasmids

A 888 bp Pst I fragment from clone pTH-13, which encodes amino acids 192 to 294 at the amino terminal part of the mature TH protein, was inserted into the Pst I site at the polylinker region of expression vectors pQE 30, 31 and 32, respectively. The insert contained pQE recombinants of three reading frames which were designated as pQE30-TH, pQE31-TH, and pQE32-TH, respectively. The TH antigen was predicted to be expressed at pQE30-TH, where the insert is in the open reading frame if it is in the right orientation. Because the pQE expression plasmids do not provide color selection of plasmids that contain inserts after ligation and transformation, we chose enzyme mapping method to identify the insert-containing clones. There are two kinds of plasmids in the transformed host cells -- the recombinated pQE and pREP4; therefore, identifying the recombinant pQE construct by enzyme mapping requires considering the cleaved fragments of two kinds of plasmids. Single usage of Pst I cleaved the pREP4 into three fragments of 2200 bp, 923 bp and 617 bp, and cleaved the pQE into a 3440 bp vector part and a 888 bp insert part. The agarose gel separation of the 923 bp fragment from the 888 bp fragment was hardly distinguishable. The restriction enzyme Bst EII, which had no cut site in pREP4 and pQE vectors, only had one cut site in the insert fragment; therefore, combining the use of Pst I and Bst EII, the clone that had distinctive fragments of 788 bp and 110 bp was the one that containd an insert in the recombinant construct.

3.4.2 Expression of the Transhydrogenase Antigen

Because the insert orientation ligated to the vector was unknown, we expressed more than four clones of each construct, pQE30-TH, pQE31-TH and pQE32-TH, respectively. As shown in Figure 3.1A, SDS-PAGE analysis of cell lysates of different constructs showed a distinctive extra large amount of protein at MW of 39-kDa, the predicted size of expressed product, only presented at lane 7 and 12, which were IPTG induced clone pQE30-TH1 and pQE30-TH3, not showed in other induced constructs nor the uninduced strains. In lane 3, the induced cell lysate of pQE16, which contains in frame insert and can express dihydrofolate reductase (DHFR) of 26-kDa as expression control, showed distinctive extra large amount of protein at MW of 26-kDa on SDS-PAGE. Western Blot analysis (Figure 3.1B) showed that only the proteins at 39-kDa in pQE30-TH1 and pQE30-TH3 cell lysates were probed by the 110-kDa protein specific antibodies (110 antibody-1). This result indicated that none of the *E. coli* bacterial protein was recognized by the 110 antibody-1. This antibody itself is a mixture of antibodies specific to TH protein and to Na^+/Ca^{2+} antiporter; therefore, we could infer that the bacteria did not contain any proteins that share antigenicity with TH or Na^+/Ca^{2+} antiporter. This phenomenon gave us the confidence that only the expressed TH antigen could be recognized by the 110 antibody-1 and the purity of the expressed protein could be confidentially sure that there is no Na^+/Ca^{2+} antiporter in it.

The growth rates of *E. coli* cultures were unaffected by the presence of the expression plasmid for TH. The time course of the expression characteristics was analyzed by checking expression levels at t = 1, 2, 3, 4 and 5 h after induction. The best expression was at 4 h after induction (Figure 3.2).

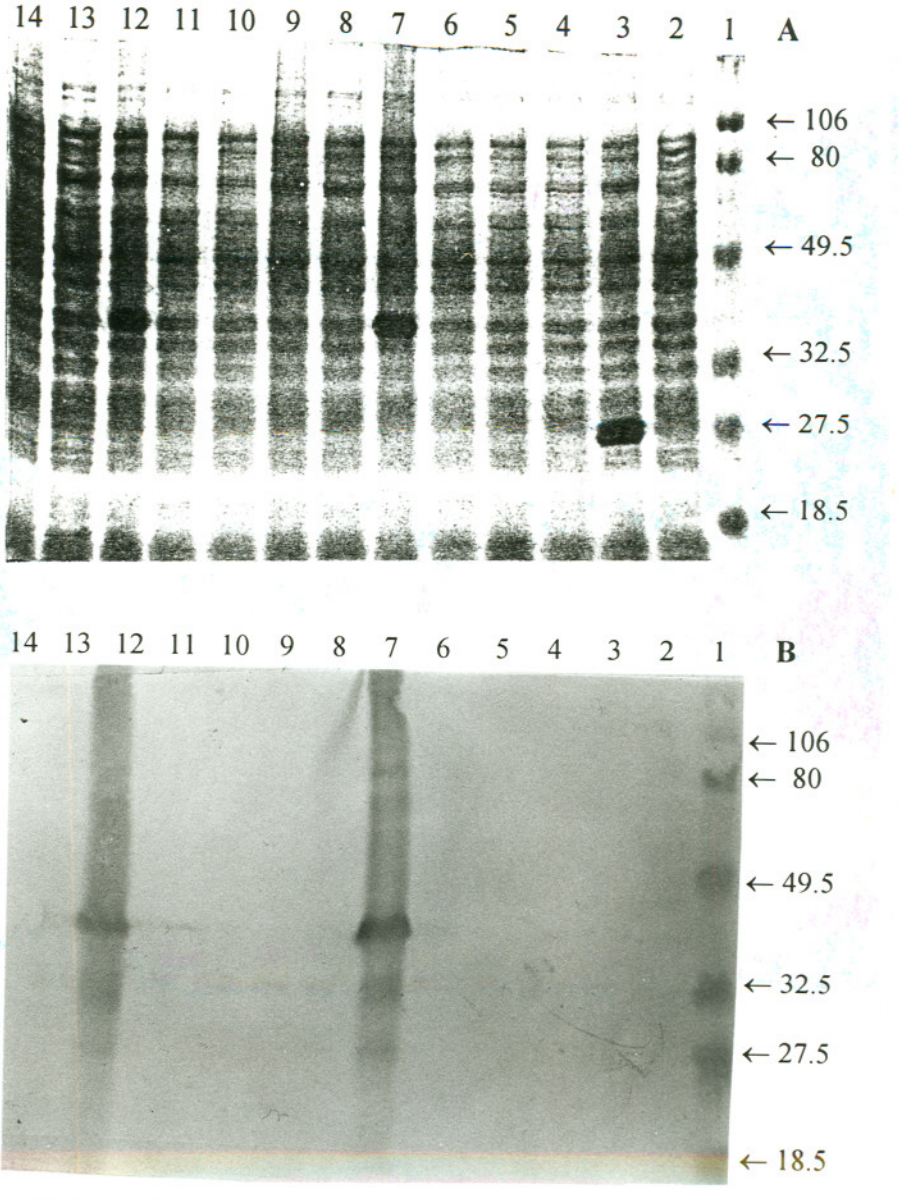

Fig 3.1 Expression of TH antigen in induced pQE30-TH construct. Lane 1, molecular weight standards; lanes 2-3, uninduced and induced cell lysate of pQE16, which contains in frame insert and can be induced to express DHFR at MW of 26-kDa on SDS-PAGE; lanes 4-5, uninduced and induced cell lysate of pQE31-TH1; lanes 6-7, uninduced and induced cell lysate of pQE30-TH1; lanes 8-9, uninduced and induced cell lysate of pQE30-TH2; lane 10, induced cell lysate of pQE30 vector; lanes 11-12, uninduced and induced cell lysate of pQE30-TH3; lanes 13-14, uninduced and induced cell lysate of pQE32-TH1. Panel A. 12% SDS-PAGE separation of the cell lysate, 20 μg/lane. Panel B. Wester blot analysis of the duplicated protein pattern as panel A, the 110 antibody-1 of titer of 1:1500 was used as first antibody.

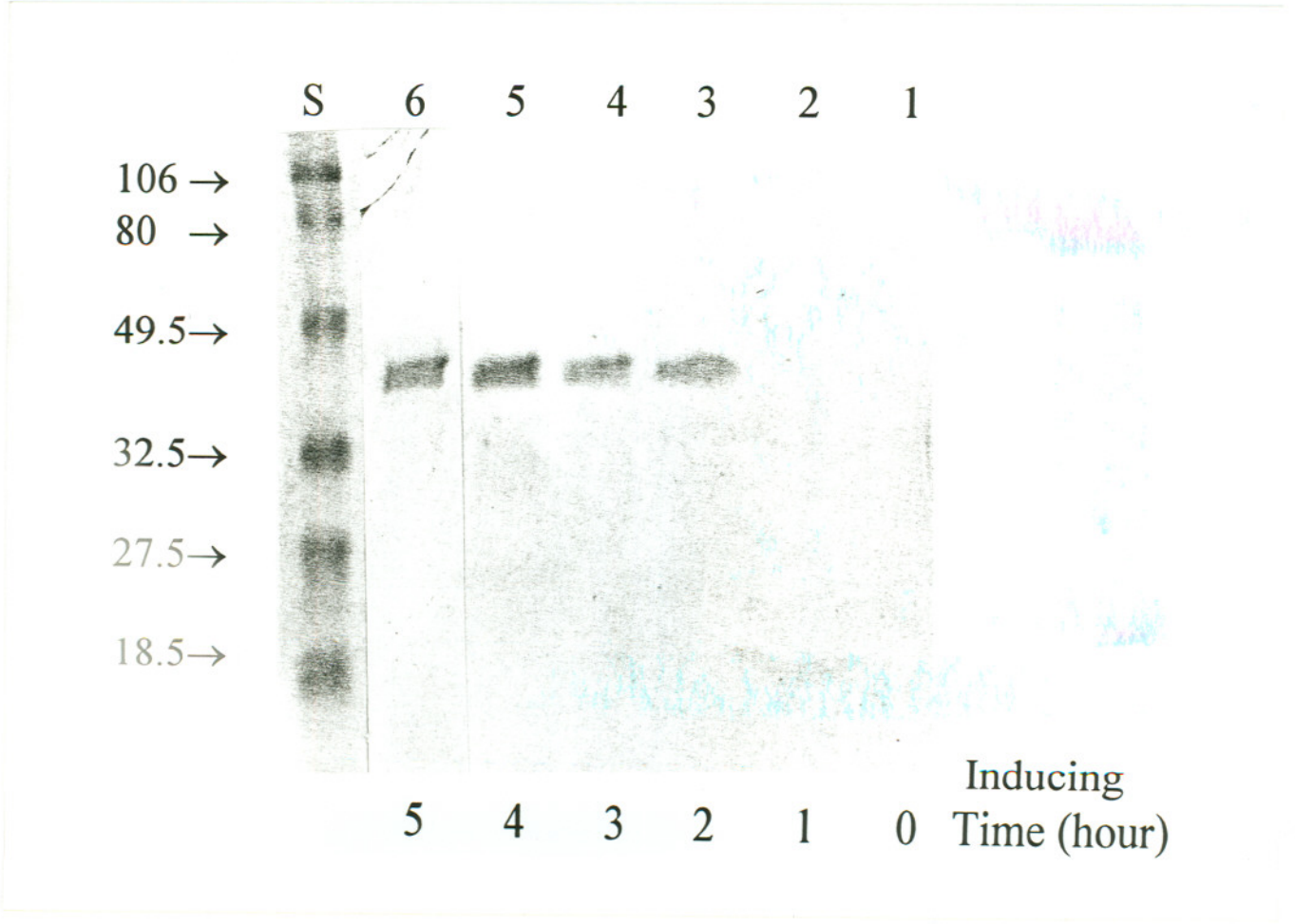

Fig 3.2 Induction time-course of TH antigen expression. TH antigen expression of clone pQE30-TH1 was induced by 2 mM IPTG. Each lane was loaded in 20 µl of the 100 µl expressed protein purified from 1 ml induced cell culture, and analyzed on 12% SDS-PAGE. Lanes 1, 2, 3, 4, 5 and 6 represented the expressed TH antigen at t = 0, 1, 2, 3, 4 and 5 h after induction. Lane S, molecular weight standard.

3.4.3 The Denatured 39-kDa TH Antigen was Identified and Purified to Homogeneity

The expressed proteins were purified under denaturing condition, where the cell lysates were loaded onto the Ni-NTA column in the presence of denaturant 6 M guanidine hydrochloride or 8 M urea. The expressed proteins that had 6×His tags at the N-terminus bound to the Ni-NTA column, and were eluted out by lower pH. Most of the expressed proteins were eluted with buffer D (pH 5.9) and some with buffer E (pH 4.5) (Figure 3.3A). The specificity of the expressed protein at 39-kDa was identified by 110 antibody-1 (Figure 3.3B). The expressed protein was designated as TH antigen. For 1 liter of induced cell culture, there were about 25 mg of expressed TH antigen to be purified.

TH antibodies against the expressed TH antigen were immunopurified. This antibody recognized the 110-kDa protein of DEAE-cellulose column purified Na^{+}/Ca^{2+} exchange active protein fraction, and probed out the 110-kDa protein at the mitochondrial membrane protein extract as shown on Figure 3.4.

3.5 The Na^{+}/Ca^{2+} Antiporter Was Separated from TH protein with Rotofor-Cell

The active DEAE-cellulose column purified 110-kDa protein fraction was subjected to Rotofor-Cell (Bio-Rad). The 20 fractions of different pH were subjected to SDS-PAGE, and the corresponded immunoblotting were tested on Western blot by TH antibodies and the 110 antibody-1, respectively. When the two antibodies were used at the same titer to TH antigen (estimated by Grid-blot that the two antibodies reacted to the same amount of expressed TH antigen with same intensity), 110 antibody-1 recognized the 110-kDa protein strongly at fractions 7 to 12, weakly at fractions 13 and 14, then strongly again at fractions 15, 16 and 17 (Figure 3.5A). On the other hand, the TH antibodies only strongly recognized the 110-kDa protein at fractions 7 to 12, but almost no reaction to fractions 13 to 17 (Figure 3.5B). The pH of fractions 1 to 6 were 2.2, 3.3, 4.3, 5.5, 6.5, 7.1, respectively. The pH of fractions 7 to 20 were indicated in Figure 3.5.

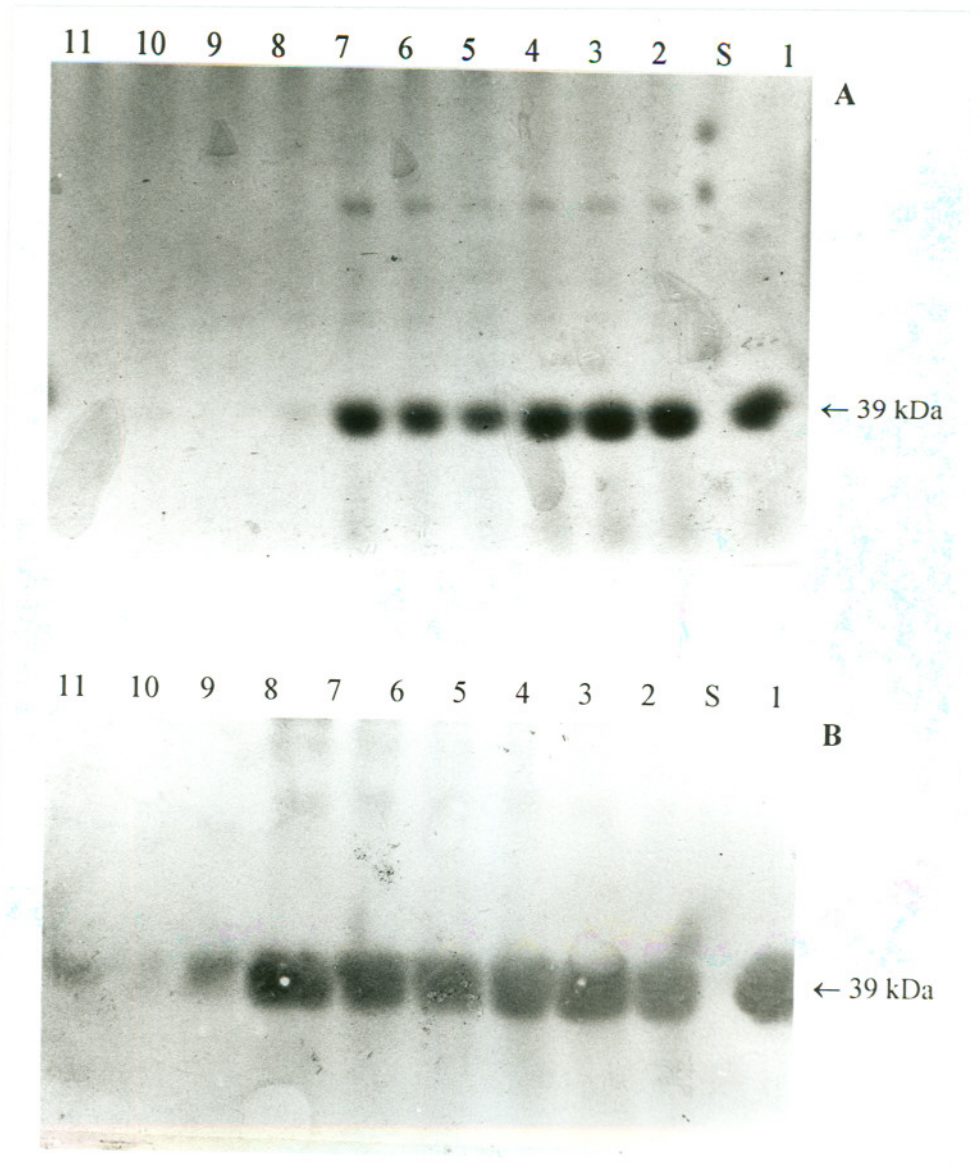

Fig 3.3 Purification of expressed TH antigen by Ni-NTA resin. Expressed TH antigen at MW of 39-kDa was purified by Ni-NTA resin under denature condition. Lane S, molecular weight standards; lane 1, induced clone pQE30-TH1 cell lysate; lanes 2-5, elution fractions eluted by Buffer D (pH5.9); lanes 6-10, elution fractions eluted by Buffer E (pH4.5); lanes 11-12, elution fractions eluted by Buffer F. Each fraction contained 5 ml of purified expressed protein, and total purification was from 1000 ml culture with 4 h induction. Panel A. 10% SDS-PAGE separation with each lane loaded in 20 µl of induced protein sample from different fractions. Panel B. Western blot analysis of the duplicated protein pattern as panel A probed with 110 antibody-1 at the titer of 1:1500.

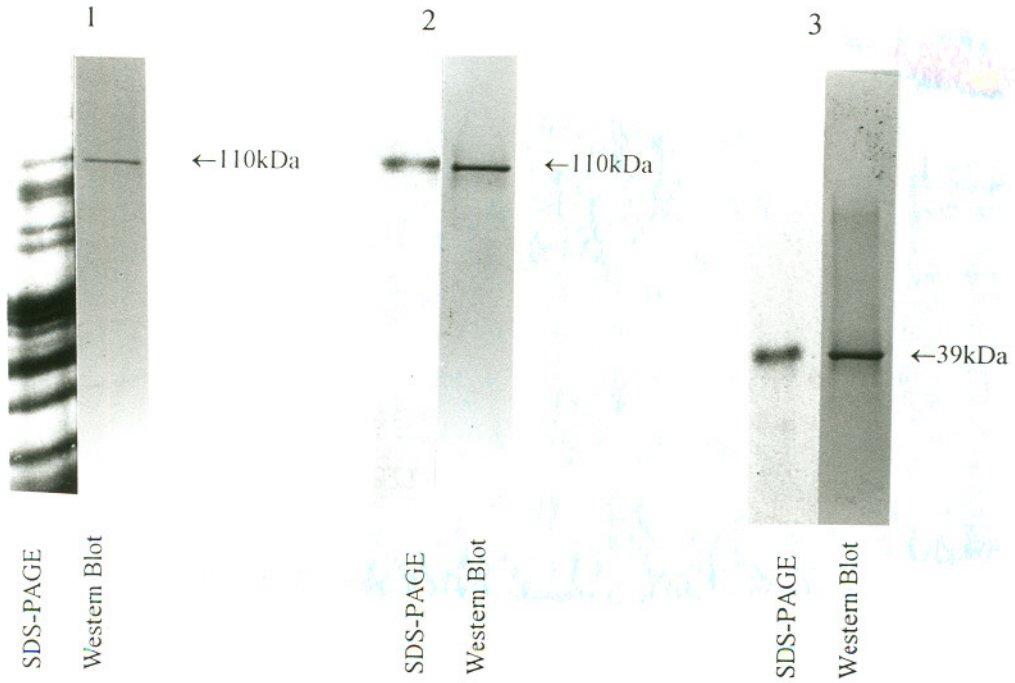

Fig 3.4 Recognition of 110-kDa protein by TH antibody. Mitochondrial membrane protein extraction (lane 1), DEAE-cellulose column purified Na^{+}/Ca^{2+} exchange active fraction (lane 2), and expressed TH antigen after Ni-NTA resin purification (lane 3) separated on Coomassie blue-stained 10% SDS-PAGE gel , and on the corresponding immunoblots probed with TH antibody at the titer of 1:2000. The polyclonal TH antibodies were immunopurified against the expressed TH antigen from 110-kDa protein raised antiserum.

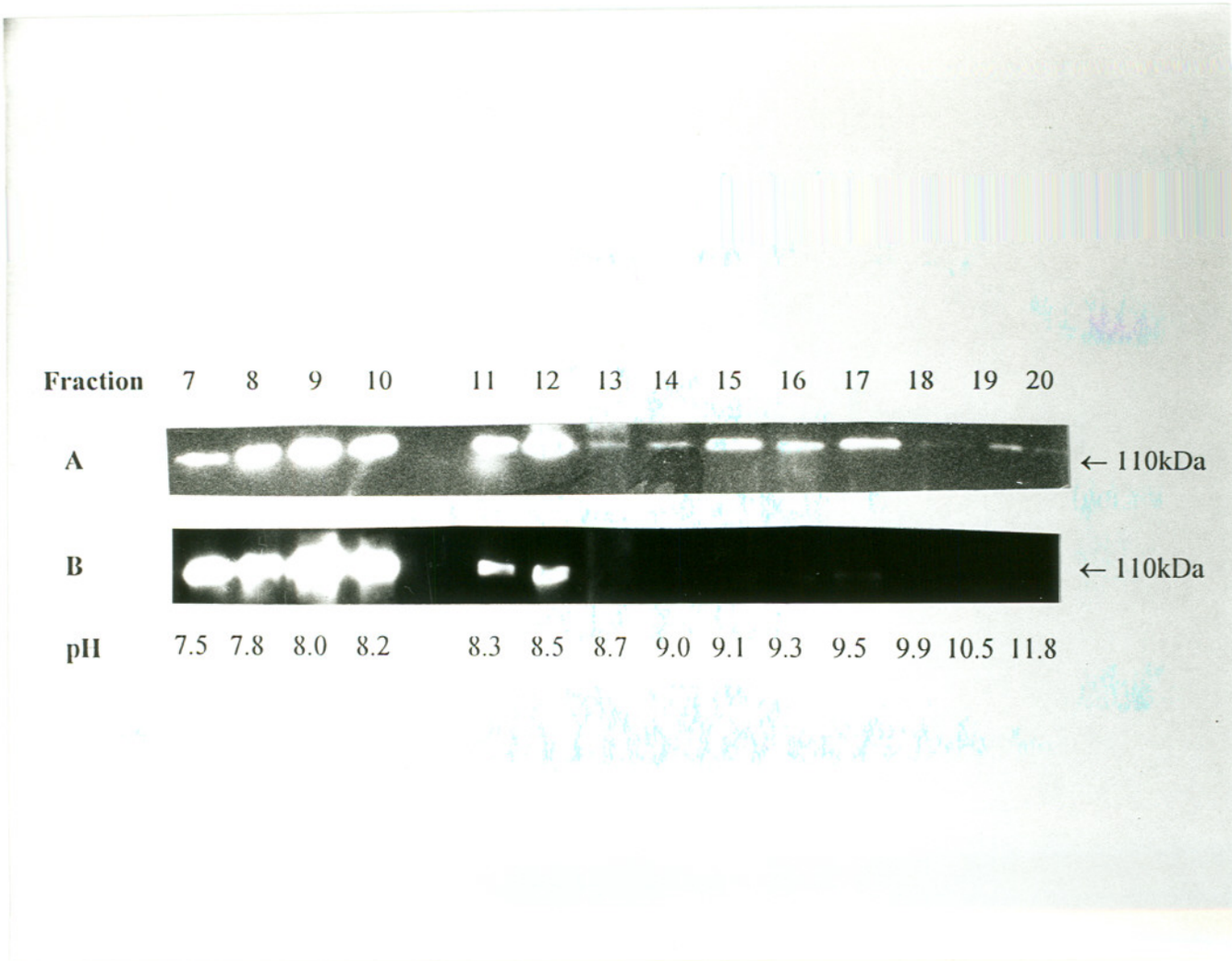

Fig 3.5 Rotofor-Cell pI separation of TH and Na^{+}/Ca^{2+} antiporter at 110-kDa. ECL Western blot of Rotofor-Cell separated 110-kDa protein. The 110-kDa protein fraction was purified by DEAE-cellulose column with high Na^{+}/Ca^{2+} exchange activity. Row A. Rotofor separated 110-kDa protein fractions probed by 110 antibody-1. Row B. Duplicated protein fractions as Row A probed by TH antibody. No.6 to 20 were the Rotofor-Cell separated fraction numbers, and the corresponding pH of each fraction was marked at button. The titers of both antibodies were adjusted to 1:1000 according to the reaction to expressed TH antigen.

Rotofor-Cell separation clearly showed that there were two proteins at the molecular weight of 110-kDa, and that the protein with the pI of about 8.1 which was recognized by both antibodies most probably was TH, while the other protein with the pI of 9.2 that was not probed by TH antibody possibly was our putative Na^+/Ca^{2+} anitporter. From this experiment, we could see that these two proteins did not focus well at one point of pI. The reason could be that these two proteins are highly hydrophobic membrane proteins and they might precipitate at or near the pH of their pI, so some of the proteins had already precipitated before they migrated and focused at the compartment with the pH of its pI. Visible protein precipitation during Rotofor focusing was quite dominant with our observation. For the 0.5 mg of 110-kDa protein in the pre-Rotofor solution, only about 100 μg of 110-kDa protein still solublized in the post-Rotofor solution; therefore, about 80% of 110-kDa protein precipitated during the Rotofor-Cell separation. In fractions 14 to 17, the fractions containing our targeted protein — 110-kDa Na^+/Ca^{2+} antiporter — the 110-kDa protein band was hardly visible on Coomassie blue stained SDS-polyacrylamide gel; in addition, this diluted sample contained 1% non-ionic detergent Triton X-100. The later handling of this sample, like concentrating and removing extra detergent, demonstrated the loss of significant amounts of our target protein. Therefore, the use of Rotofor-Cell to accumulate sub-milligram quantities of concentrated Na^+/Ca^{2+} antiporter for further experiments proved not very practical.

3.6 The Na^+/Ca^{2+} Antiporter was Significantly Enriched by Negative Immuno-purification

The advantages of immunopurification over Rotofor separation are that mixtures of 110-kDa protein fractions can be concentrated before purification, which reduces the loss of our target protein; besides, the immunopurified Na^+/Ca^{2+} antiporter fraction can be used directly for further purposes, such as amino acid analysis and antibody purification without

the necessity of removing denaturants like urea and guanidine chloride in liquid isoelectric focucing.

The coupling efficiency of the TH antibody to an AminoLink column was 86%. The concentrated 110-kDa protein sample contained 12.6% Triton X-100. Such a high concentration of non-ionic detergent interferes with the antigen-antibody reaction, as our experiment indicated that the specific antigen-antibody binding decreased dramatically with higher than 2% Triton X-100 (data not shown). Hence, extra Triton must be eliminated to keep its concentration around 1%. 3.6 g detergent absorber gel removed 12.6% Triton X-100 to 1.5% in the 5 ml concentrated 110-kDa protein fraction sample. The detergent absorber gel proved to be an easy, quick and efficient method to eliminate non-ionic detergent, but this method also was accompanied by the loss of certain amounts of protein when detergent absorber gel directly contacted the protein sample. In our case, there was about 25-30% protein loss.

The resultant flow-through contained the proteins that did not bind to the immobilized TH antibodies on the AminoLink gel. The proteins that bound to the TH antibody immobilized column were eluted by lowing the pH. Western blot analysis of each fraction was checked by the same titered 110 antibody-1 and TH antibody, respectively. The antibody titers were adjusted by Grid-blot that the two antibodies reacted to the same amount of expressed TH antigen with similar density. In Figure 3.6, 110 antibody-1 strongly reacted to the 110-kDa protein at flow-through and wash fractions (Figure 3.6A), but the TH antibody showed a much weaker reaction to the same amount of 110-kDa protein at flow-through and wash fractions (Figure 3.6B), while both antibodies reacted to the same amount of 110-kDa protein at elute fractions (supposed to contain only TH protein of 110-kDa) of the same intensity. This result also revealed the existence of two proteins at 110-kDa. The difference of the same protein sample in flow-through reacted to two different kinds of antibodies indicated that the resultant flow-through and wash fractions contained most of our putative Na^{+}/Ca^{2+} antiporter and little TH.

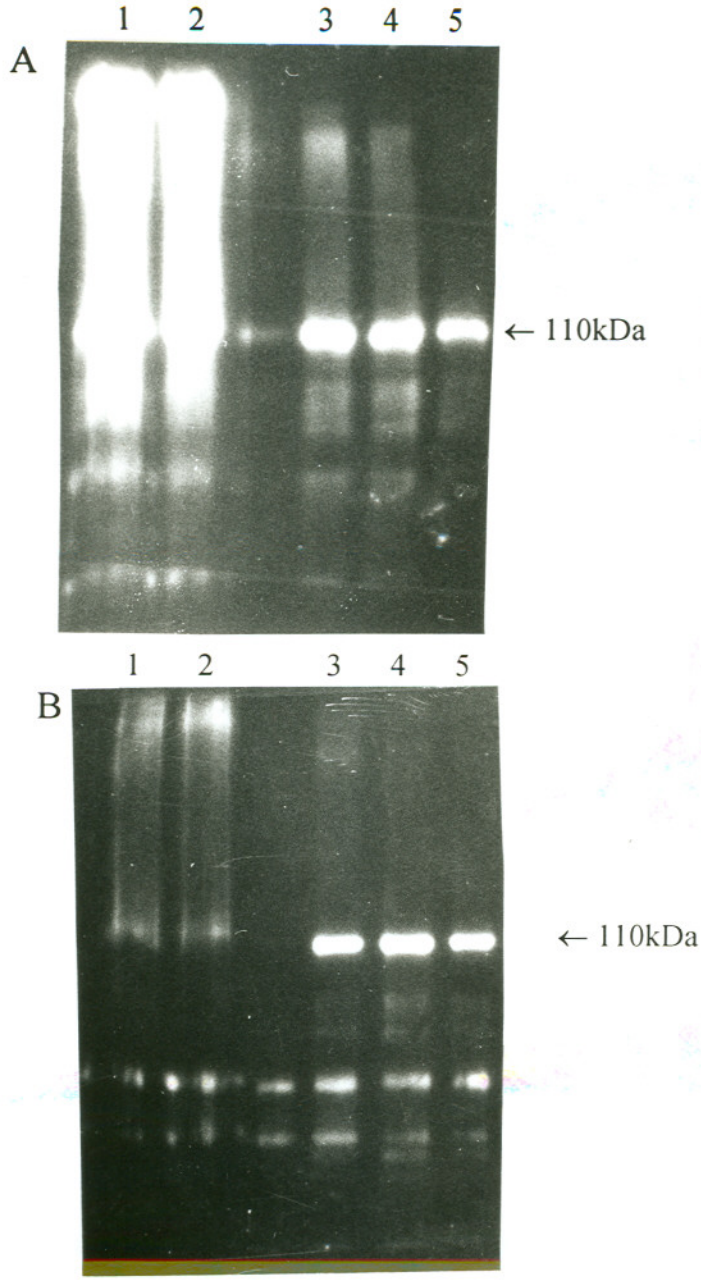

Fig 3.6 AminoLink gel negative purification removing TH protein from Na^+/Ca^{2+} antiporter. The DEAE-cellulose partial purified Na^+/Ca^{2+} exchange active protein fractions were loaded into the TH antibody immobilized AminoLink column. The duplicated immunoblot of resultant flow-through fraction (lane 1), first 1 ml wash fraction (lane 2) and elution fractions (lanes 3-6) were probed with 110 antibody-1 (panel A) and TH antibody (panel B). The titers of both antibodies were adjusted to 1:1000 according to the reaction to expressed TH antigen.

This point of view is also supported by Grid-blot results, which were probed by the antibodies (110 antibody-2) against the 110-kDa protein band of flow-through and wash fractions of the AminoLink affinity column. Comparing the 110 antibody-2 with TH antibody (Figure 3.7), we observed that when the titers of both antibodies were adjusted to react to the same amount of TH protein at approximately the same density (panels D and C), the 110 antibody-2 probed the active 110-kDa protein fraction much stronger than the TH antibody to the same amount of 110-kDa protein (panels B and A). This result indicated that the 110-kDa protein contained another protein (the putative Na^+/Ca^{2+} antiporter) besides TH, and that the 110 antibody-2 contained antibodies specific to the putative Na^+/Ca^{2+} antiporter in the 110-kDa protein fraction mixture. This phenomenon was also demonstrated by Western blot in Figure 3.8. The 110-kDa protein of DEAE-cellulose purified Na^+/Ca^{2+} exchange active fraction also showed that it contained another protein, besides TH, which could be recognized by specific antibodies in 110 antibody-2.

In comparing the specificity of 110 antibody-1 to 110 antibody-2, when both antibodies recognized the same amount of DEAE-cellulose purified 110-kDa protein which contained mixtures of TH and putative Na^+/Ca^{2+} antiporter at the same intensity (Figure 3.9, panels A and B), 110 antibody-2 probed expressed TH protein much weaker than did 110 antibody-1 to the same amount TH protein (panels D and C). This result revealed that 110 antibody-2 contained a much higher ratio of Na^+/Ca^{2+} antibody : TH antibody than 110 antibody-1.

3.7 Inhibition of the Mitochondrial Na^+/Ca^{2+} Antiporter by Antibodies against the 110-kDa protein

Previous data from our lab (Li et al., 1992) indicated that the 110 antibody-1 (antibodies against the 110-kDa DEAE-cellulose purified protein) inhibited Na^+/Ca^{2+} antiport activity, but preimmune serum as a control did not. This time, we used the 110 antibody-2 (antibody against the immunopurified 110-kDa protein) incubated with the

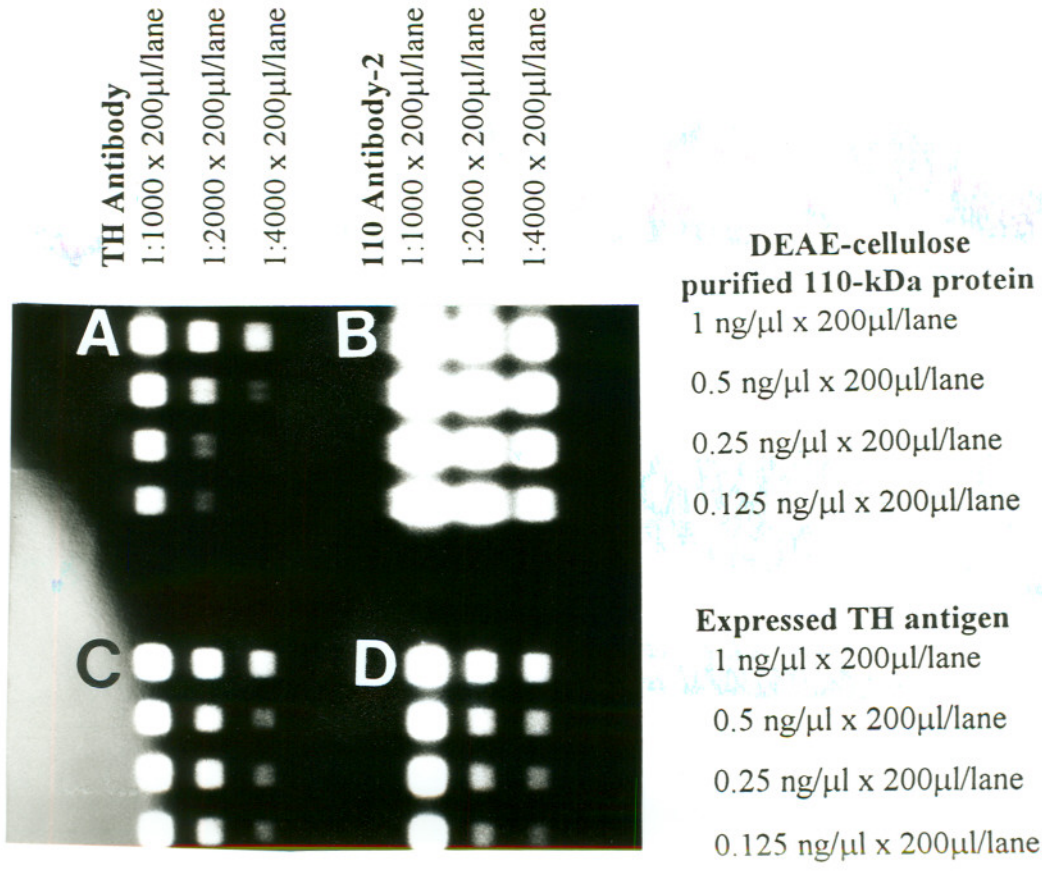

Fig 3.7 Grid Blot comparison of 110 antibody-2 with TH antibody. Rows were stepped concentrations of DEAE-cellulose purified 110-kDa protein fraction and expressed TH antigen. Lanes were stepped titers of TH antibody and 110 antibody-2. Panel A, B, reactions of 110-kDa protein to TH antibody and 110 antibody-2 respectively. Panel C, D, reactions of TH antigen to TH antibody and 110 antibody-2 respectively.

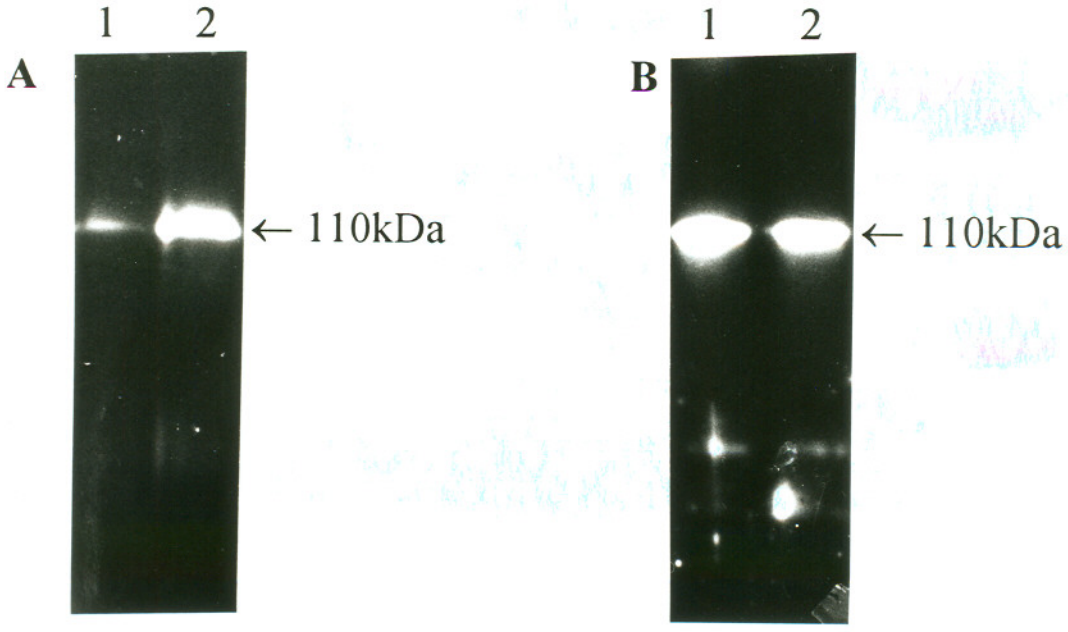

Fig 3.8 Western-blot comparison of 110 antibody-2 with TH antibody. Lane 1, TH protein of 110-kDa eluted from AminoLink Column; lane 2, DEAE-cellulose column purified 110-kDa protein fraction with Na^{+}/Ca^{2+} exchange activity. Panel A , ECL Western Blot probed by 110 antibody-2. Panel B, the first antibody is TH antibody.

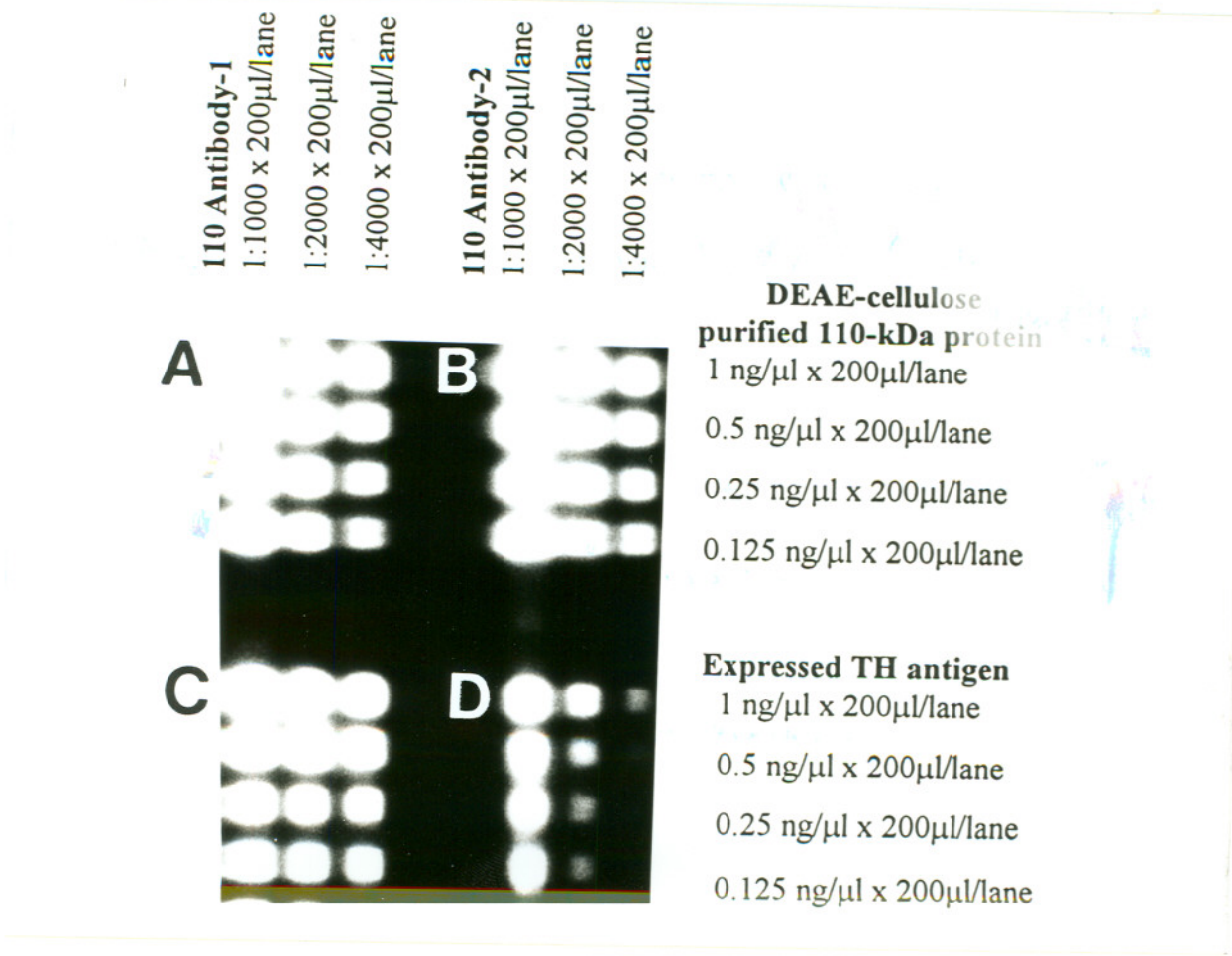

Fig 3.9 Grid-blot comparison of 110 antibody-1 with 110 antibody-2. Rows were stepped concentrations of DEAE-cellulose purified 110-kDa protein fraction and expressed TH antigen. Lanes were stepped titers of 110 antibody-1 and 110 antibody-2. Panels A and B, reactions of active 110-kDa protein to 110 antibody-1 and 110 antibody-2, respectively. Panels C and D, reactions of expressed TH antigen to 110 antibody-1 and 110 antibody-2, respectively.

native protein to determine whether they would inhibit transport. Antibody immuno-purified to the expressed TH antigen was used as a control. The antibody titers were adjusted by Grid-blot with these two antibodies reacted to the same amount of 110-kDa Na^+/Ca^{2+} exchange active protein fraction with same intensity. As seen in Figure 3.10, 110 antibody-2 inhibited Na^+-dependent Ca^{2+} uptake (curve A), while TH antibody did not show any inhibition on the Na^+/Ca^{2+} antiporter activity (curve B). These data indicated that 110 antibody-2 contained antibodies that specifically bound to Na^+/Ca^{2+} antiporter and inhibited its activity in our reconstitution system, while the TH antibody, although it exited in the 110 antibodies, did not inhibit the Na^+/Ca^{2+} antiporter. From these data, we could conclude that the 110-kDa protein band contained a mixture of two proteins, Na^+/Ca^{2+} antiporter and TH. The Na^+/Ca^{2+} exchange activity was due to the putative Na^+/Ca^{2+} antiporter protein, not the TH.

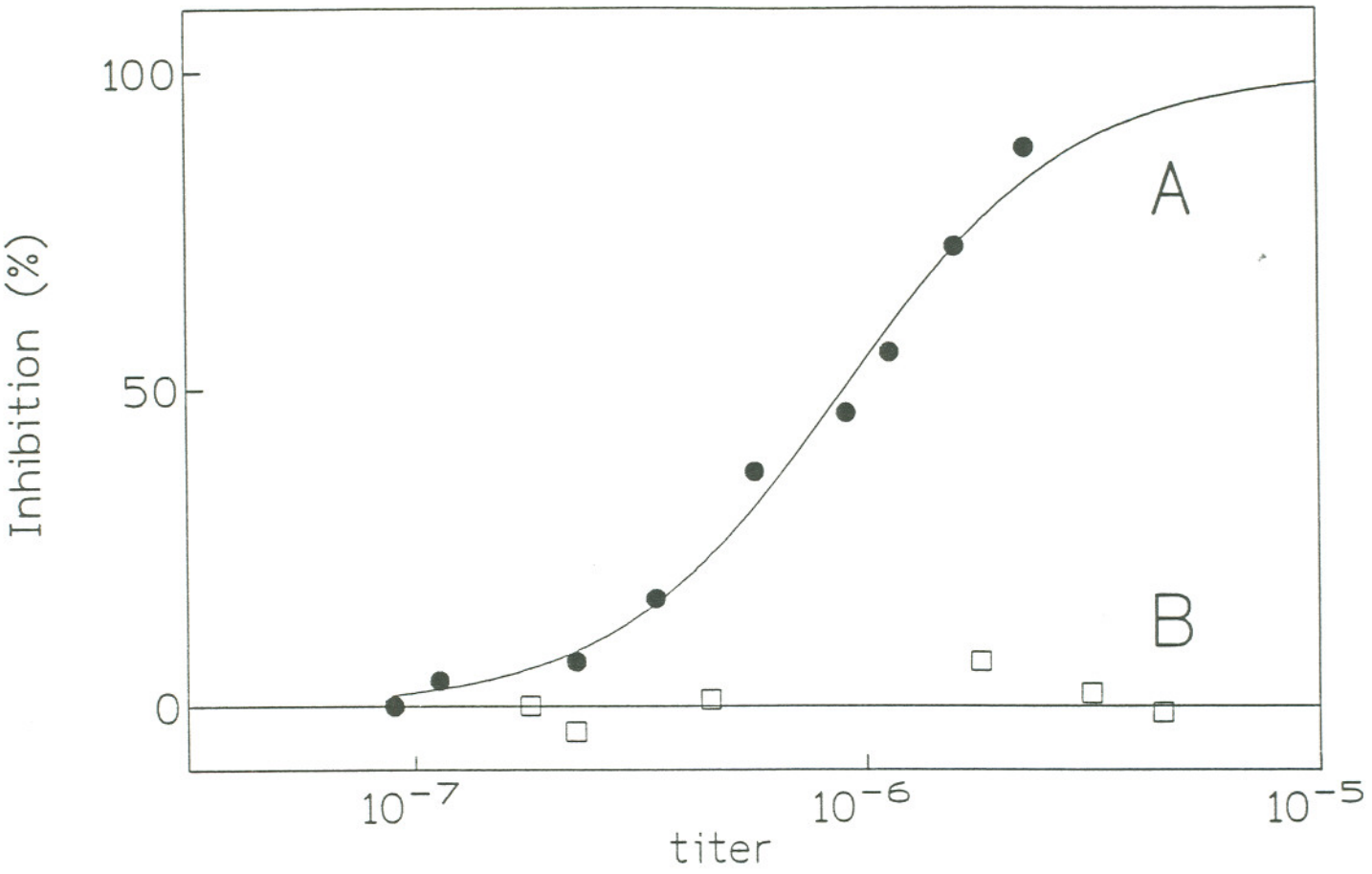

Fig 3.10 Antibody inhibition on Na^+/Ca^{2+} exchange. Dose-response curve of antibody inhibition of Na^+-dependent Ca^{2+} efflux. Na^+/Ca^{2+} exchange activity was reconstituted into proteoliposomes. An aliquot of proteoliposomes was incubated on ice with 110 antibody-2 or with TH antibody for 60 min before measurement. Trace A, effect of 110 antibody-2. Trace B, effect of TH antibody. Antibody titers were adjusted by Grid-blot of the two antibodies reacted to the same amount of active 110-kDa protein with same intensity.

Chapter 4
Discussion

4.1 Challenge in Purification of Na^+/Ca^{2+} Antiporter Protein

As our interest in investigating the molecular mechanisms responsible for the cellular function of mitochondrial Na^+/Ca^{2+} antiporter has increased, the purification of Na^+/Ca^{2+} antiporter has become a fundamental step in approaching the molecular study of this protein. The challenge, however, is that our target protein is an integral membrane protein which is highly hydrophobic and known as difficult to purify. Until now, there has been no specific nor very effective way to purify membrane protein. The methods available for the purification of membrane proteins are basically the same as those employed to purify water-soluble, nonmembrane-associated proteins.

The insolubility of membrane proteins appears to make it difficult to apply these methods successfully. Integral membrane proteins consist of hydrophilic regions exposed to the aqueous environment and hydrophobic regions embedded in the lipid phase. These proteins must often be associated with lipids or detergents to keep them solubilized in aqueous solution. Therefore, in order to purify the Na^+/Ca^{2+} antiporter by ion-exchange chromatography, the mitochondrial inner membrane proteins must be removed from the lipid bilayer and individually dispersed into solution with the help of amphiphilic detergent. In our case, with many trial experiments and overall consideration, the non-ionic detergent Triton X-100 is optimal to purify the active fractions which show the highest reconstitutative Na^+/Ca^{2+} exchange activity.

The sarcolemmal membrane Na^+/Ca^{2+} antiporter has proven to be a difficult protein to isolate as documented while it was worked out finally by Philipson (Philipson et al., 1988). Our early purification steps share a lot of similarity with Philipson's method to

purify the sarcolemmal Na^+/Ca^{2+} antiporter. Both methods use the non-ionic detergent Triton X-100 to solubilize the membrane proteins, and DEAE ion-exchange chromatography is used to fractionate the solubilized proteins. Their high quality purification leads to cloning the exchanger successfully. We used similar techniques in early steps and followed by functional assays of the reconstitution system. However, this purification misled the cloning work and revealed a contaminant TH protein which shares the same molecular weight with the putative Na^+/Ca^{2+} antiporter. Therefore, separating Na^+/Ca^{2+} antiporter from TH contamination is the key point in successful cloning.

4.2 Evidence for the Existence of Two Proteins at 110-kDa band

Our previous data indicated that the Na^+/Ca^{2+} exchange activity corresponded to the inner mitochondrial membrane protein at 110-kDa protein in the wash fraction of DEAE chromatography (Li et al., 1992). The result that the 110-kDa protein specific antibodies probed out mitochondrial nicotinamide nucleotide transhydrogenase (TH) cDNA clone indicated that the 110-kDa protein band contained TH protein. TH protein is also 110-kDa in size (Yamaguchi & Hatefi, 1988), the same as the identified Na^+/Ca^{2+} antiporter. They both are transmembrane proteins integrated to the inner mitochondrial membrane. The questions that arise here are: are there two proteins at 110-kDa, the TH and the putative Na^+/Ca^{2+} antiporter, or is there only one protein at 110-kDa — the TH? Does TH have multiple functions working as a transhydrogenase and also as a cation transporter?

Our identification of another protein, the putative Na^+/Ca^{2+} antiporter, presenting at 110-kDa rests on the following evidence. (i) The pI separation with liquid phase isoelectrical focucing by Rotofor-Cell implied the existence of two proteins at 110-kDa, one with the pI 8.1 is probed by TH antibody and by antibodies against the mixture of 110-kDa proteins (110 antibody-1), another with pI 9.2 is only recognized by 110 antibody-1, but not the TH antibody (Figure 3.5). (ii) The negative purification of

putative Na^+/Ca^{2+} antiporter protein with TH antibody coupled affinity column also suggested that the 110-kDa protein in the resultant flow-through fraction was specifically recognized by 110 antibody-1 as shown in Figure 3.6. Although there was a little TH protein left which could be probed out by TH antibody, the amount of leftover TH protein dramatically decreased (see Figure 3.6). (iii) Antibodies against the negative purified 110-kDa protein (110 antibody-2) confirmed and amplified the phenomenon of the existence of two proteins at 110-kDa, as resolved in Figures 3.7, 3.8, and 3.9. (iv) The antibody inhibition in reconstitution system indicated that the 110-kDa protein specific antibodies (110 antibody-1, 110 antibody-2) inhibited the Na^+-dependent Ca^{2+} uptake, while the TH antibody did not show any inhibition of the Na^+/Ca^{2+} antiporter activity as shown in Figure 3.10. This data also indirectly implied that the TH protein is not responsible for the Na^+/Ca^{2+} exchange activity.

4.3 The Reasons for TH Protein Copurified with Na^+/Ca^{2+} Antiporter

The solubilization of membrane protein can not be carried out unless some detergent is applied. However, the interaction of detergent with protein is not specific at all. Detergent micelles may form complexes with not only one hydrophobic protein, but with multiproteins non-specifically. The non-ionic detergent, Triton X-100 in this case, is less effective in dissociating protein complexes than ionic detergents. Hence, the co-purification of TH protein in our Na^+/Ca^{2+} exchange active fraction might be due to association of TH and Na^+/Ca^{2+} antiporter in a protein-detergent-micelle complex, which actually behaves like single molecule in chromatography column. Although other mitochondrial membrane proteins can be involved in the complex also and are copurified with the Na^+/Ca^{2+} antiporter, it is not a problem because they can be separated by SDS buffer while running SDS gel electrophoresis. However, TH protein shares the same molecular weight as the putative antiporter protein, that is, SDS-PAGE would not help in separation.

4.4 The Evidence for Separation of the Na^+/Ca^{2+} Antiporter Protein from TH Protein Contamination

As we did rationales in the Results section, an effective separation has to be approached by and relied on one of four factors: putative Na^+/Ca^{2+} antiporter protein, TH protein, and two corresponding antibodies. The factors as diagnosis means that to monitor and follow the separation have to be guaranteed pure . We carefully considered all techniques available in protein chemistry and immunology and none of them can avoid uncertainties because of contaminantion and non-specific adsorption. However, molecular cloning technology provides an ideal approach to accept this challenge and solve this problem. A piece of antigenic peptide selected from TH protein was successfully expressed in bacteria *E. coli* (Figure 3.1) and purified to homogeneity with his-tag affinity purification technology (Figure 3.3). Thus, we first obtained guaranteed pure TH protein antigen without any Na^+/Ca^{2+} antiporter protein contamination with molecular cloning technology.

Based on this success, the separation proceeded further with immunological affinity column, which offered the highest specificity in chromatography purification. As we discussed in Results, negative purification is the most feasible method of separating the antiporter from TH contamination. Using this method, we obtained the fraction with enriched Na^+/Ca^{2+} antiporter, although there was always a little TH protein left. The main reason for incomplete separation might be the influence of the non-ionic detergent, Triton X-100.

Detergent is a necessary element to keep our target protein in soluble form, but its presence influences the behavior of our target protein, like chromatography retention, antigen-antibody interaction, and migration on SDS-PAGE. To overcome this problem, we need to keep a certain concentration of detergent in our protein solution to maximize the concentration of solublized membrane protein and to minimize the protein behavior

interference. From our experience, this balanced point was to keep Triton X-100 at around 1%.

Before negative purification, the partially purified Na^+/Ca^{2+} exchange active protein fraction needed to be concentrated; meanwhile, the Triton X-100 concentration was increased proportionally. Since the CMC of Triton X-100 is very low (only 0.2-0.9 mM in 0.05 M Na^+ solution), removing extra Triton X-100 using dialysis is not practical. We also tried precipitating the proteins with trichloroacetic acid, then changing ionic-detergent to solubilize the precipitated proteins, but the resolubilization was poor because of the high hydrophobicity of our target protein. Hydrophobic adsorption (chromatography) is a efficient method for the removal of extra Triton X-100, but certain amounts of protein sample are also adsorbed to the matrix, yielding lower recovery at this step.

With negative purification of the Na^+/Ca^{2+} antiporter through TH antibody immobilized column chromatography, the resultant flow-through fraction always contains a little TH protein, although the column binding sites for TH protein are far more than saturation. The incomplete purification may be due to the existence of Triton X-100, which form very heterogeneous detergent-protein micelles. Some conformation of protein or the position of protein in micelles hide their antigenic part and interfere with the antigen-antibody binding.

4.5 Future Experiment

The long-term goal of our research on the mitochondrial Na^+/Ca^{2+} antiporter is to clone this carrier and to elucidate the molecular mechanisms of this transporter protein. The primary approach toward this aim is to get the right probe and to isolate the Na^+/Ca^{2+} antiporter clone from a library. Our efforts on separating the Na^+/Ca^{2+} antiporter from TH contamination were directed towards this goal. Our lab is now pursuing two avenues towards obtaining the Na^+/Ca^{2+} antiporter clone. One is to approach positive clones by

immunoscreening a cDNA library with polyclonal antibodies as probe. The 110 antibody-2, which is against the negative purified 110-kDa protein in which the putative Na^{+}/Ca^{2+} antiporter protein is highly enriched, will be used as probe to screen an expression cDNA library for positive clones. Another approach is to screen a cDNA library with degenerated primers designed against the peptide sequences. This approach requires a great deal of negatively purified Na^{+}/Ca^{2+} antiporter protein, which became realistic after established the methodology in this study. The effort towards to both approaches is underway in our laboratory.

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BIOGRAPHICAL SKETCH

The auther was born on February 19, 1966, in Shanghai, the People's Republic of China. In 1983, she started her college education at the Department of Medicine, Beijing Medical University, where she was awarded the Outstanding Student Award in 1986. She received her Bachelor of Medicine in 1989, then accepted a residency position at the Peking Union Medical College Hospital. She began her graduate study at the Medical College of Ohio in the fall of 1992, then transferred to the Oregon Graduate Institute in the spring of 1993, under the supervision and direction of Dr. Keith Garlid. While in Dr. Garlid's lab, she worked on the purification and cloning of the mitochondrial Na^+/Ca^{2+} antiporter.