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THE EFFECT OF METAL IONS ON MITOCHONDRIAL PYRIDINE DINUCLEOTIDE TRANSHYDROGENASE

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Summary

Bovine heart submitochondrial particle transhydrogenase is inhibited by cations in a concentration and pH-dependent manner, and non-energy-linked transhydrogenation is inhibited to a greater extent by metals than the energy-linked reaction. The inhibition of the enzyme by Mg^{2+} is competitive with the NADP substrate and non-competitive with the NAD substrate. Mg^{2+} stimulates inactivation of the enzyme by 5,5′-dithiobis(2-nitrobenzoic acid), and protects against thermal and proteolytic inactivation. This suggests that Mg^{2+} binding in the NADP site alters transhydrogenase to a more thermostable conformation, which is less susceptible to attack by trypsin and more reactive with 5,5′-dithiobis(2-nitrobenzoic acid). Other cation inhibitors mimic Mg^{2+} in these properties. The order of effectiveness of the inhibitors tested is $La^{3+} > Mn^{2+} > Ca^{2+} \simeq Mg^{2+} > Sr^{2+} > Na^{+} \simeq K^{+}$. This order is described by the Irving-Williams order for the stability of metal-ligand complexes, suggesting that carboxylates or amines may comprise the inhibitory cation binding site.

Introduction

Mitochondrial inner membrane-bound pyridine dinucleotide transhydrogenase catalyzes a reversible transfer of a hydride ion equivalent between intra-

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Abbreviations: $AcPyAD^{+}$, oxidized 3-acetylpyridine adenine dinucleotide; DPCC, diphenylcarbamyl-chloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DTNA, 6,6′-dithiodinicotinic acid; DTP, 2,2′-dithiodi-pyridine; FDS, formamide disulfide dihydrochloride; PCMB, *p*-chloromercuribenzoic acid; PCMBS, p-chloromercuriphenylsulfonic acid; TDET, 2,2′-thiodiethanethiol; thio-$NADP^{+}$, oxidized thionicotin-amide adenine dinucleotide phosphate.

mitochondrial NADH and $NADP^+$ (Eqn. 1) [1—3].

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH \quad (1)$$

Energization of submitochondrial membranes by oxidation of respiratory chain substrates or ATP hydrolysis enhances the forward reaction rate several fold [1—3] and increases the apparent equilibrium constant from 1 to nearly 500 [4]. Recently, Höjeberg and Rydström [5] and Earle et al. [6] reconstituted purified bovine heart transhydrogenase in phosphatidylcholine liposomes and demonstrated that transhydrogenation between NADPH and NAD^+ is linked to the formation of an electrochemical potential and a pH gradient across the membrane, respectively. Hence, the energy-linked transhydrogenase reaction may be described by coupling proton translocation across the membrane to the oxidation-reduction reaction (Eqn. 2), where c and m represent the cytosolic and matrix sides of the inner mitochondrial membrane.

$$nH_c^+ + NADH + NADP^+ \rightleftharpoons nH_m^+ + NAD^+ + NADPH \quad (2)$$

That separate NAD and NADP binding domains form the transhydrogenase active site is illustrated by (a) stereospecific transfer of hydrogen from the 4A locus of NADH to the 4B locus of NADPH [7], (b) identification of site-specific inhibitors competitive for binding with either NAD or NADP substrates [8], and (c) kinetics which indicate that the reaction proceeds through a ternary complex, where in both directions the NAD substrate binds prior to the NADP substrate [8,9].

Divalent metal ions, including Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mn^{2+}, inhibit non-energy-linked transhydrogenation [4,10—12]. Mg^{2+} has been reported to inhibit the energy-linked reaction to a greater degree than the non-energy-linked reaction [11,13], with the extent of inhibition increasing with increasing medium pH [13]. The nature of cation inhibition is not established. Hommes [11] reported Mg^{2+} to be a competitive inhibitor of NAD^+ binding, but a non-competitive inhibitor of NADH binding. Rydström [8], however, was unable to conclude whether the inhibitory Mg^{2+} binding site is in the NAD domain, the NADP domain, or elsewhere on the enzyme.

Materials and Methods

Bovine heart mitochondria and submitochondrial particles were prepared as described previously [14].

Sulfhydryl group modifications. Submitochondrial particles (0.3—0.5 mg protein) were preincubated at indicated pH under non-energized conditions at 22°C in a medium (0.61 ml) consisting of 20 mM Tris/acetate plus the sulfhydryl reagent and other additions as indicated. The preincubation medium for energized membranes contained in addition 7.5 mM sodium succinate and 3 μg oligomycin. The modified membranes were assayed immediately for reverse non-energy-linked transhydrogenase activity by diluting the preincubation mixture to 3 ml with the assay mixture.

Thermal inactivation. Submitochondrial particles (0.4 mg protein) were preincubated at 53°C for 110 s in a medium (0.61 ml) consisting of 20 mM Tris/acetate, pH 7.5, plus indicated additions. The mixtures were taken to 0°C in

an ice/water bath and assayed for residual reverse non-energy-linked transhydrogenase activity after warming to room temperature. Controls were performed identically by preincubation at 22° C.

Proteolytic inactivation. Submitochondrial particles (0.4 mg protein) were preincubated for 1 min at 22° C in the presence of 30 μg DPCC-treated trypsin, 20 mM Tris/acetate, pH 7.5, and where indicated 7.5 mM sodium succinate, 3 μg oligomycin, 5 mM $MgCl_2$, and 100 μg trypsin inhibitor. The reaction was terminated by immediately diluting the preincubation mixture (0.61 ml) to 3 ml with the reverse non-energy-linked transhydrogenase assay medium.

Transhydrogenase assay. Non-energy-linked reverse transhydrogenase activity was assayed according to Blazyk et al. [12] at 22° C in a medium (3 ml) containing submitochondrial particles (0.4—0.6 mg protein), 100 mM Tris/acetate, pH 6.8, 0.5 μM rotenone, 150 μM NADPH, 190 μM $AcPyAD^+$, and other indicated additions.

In kinetic studies on the forward transhydrogenase reaction the assay mixture (3 ml) contained 0.23 mg submitochondrial particle protein, 100 mM Tris/acetate, pH 7, 0.5 μM rotenone, 120 μM NADH, and 50 μM thio-$NADP^+$. The formation of thio-NADPH was monitored as an increase in absorbance at 395 nm assuming a millimolar extinction coefficient of 11.3 cm^{-1} [15]. The reverse reaction medium was identical except 0.04 mg protein was assayed and 120 μM $AcPyAD^+$ and 120 μM NADPH were the substrates. Formation of AcPyADH was determined as described for the standard assay. The K_m values obtained were 15 μM NADH, 1.5 μM thio-$NADP^+$, 15 μM NADPH, and 12 μM $AcPyAD^+$.

Pyridine dinucleotides, with the exception of $AcPyAD^+$ which was prepared by the method of Kaplan and Ciotti [16], were purchased from P-L Biochemicals. Antimycin A was from Calbiochem. 5,5′-Dithiobis(2-nitrobenzoic acid), *p*-chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid, 2,2′-dithiodipyridine, *N*-ethylmaleimide, formamidine disulfide dihydrochloride, iodoacetamide, iodoacetate, soybean trypsin inhibitor, and DPCC-treated trypsin were from Sigma Chemical Co. 6,6′-Dithiodinicotinic acid and 2,2′-thiodiethanethiol were purchased from Aldrich Chemical Co. All reagent grade inorganic salts were products from Fisher Scientific.

Results

Cation inhibition of transhydrogenase

At pH 7.0 the inhibition of bovine heart submitochondrial reverse non-energy-linked transhydrogenase is a function of the concentration of the divalent cations, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Sr^{2+}, as well as the trivalent ion, La^{3+} (Fig. 1). Cation concentrations giving 50% inhibition of transhydrogenase activity were approximately 25 mM Sr^{2+}, 20 mM Ca^{2+} or Mg^{2+}, 10 mM Mn^{2+} and 100 μM La^{3+}. Rydström et al. [13] reported that Mg^{2+} inhibition of transhydrogenase increases with increasing pH. A similar inhibitory pattern is seen with Ca^{2+}, Mn^{2+}, and Sr^{2+} (Fig. 2). With 200 μM La^{3+}, inhibitor effectiveness increases similarly from pH 5.5 until precipitates of $La(OH)_3$ form at about pH 8.0. When the effects of K^+, Na^+, and Tl^+ are tested at a pH particularly favorable for divalent cation inhibition (pH 7.9) it is evident that monovalent

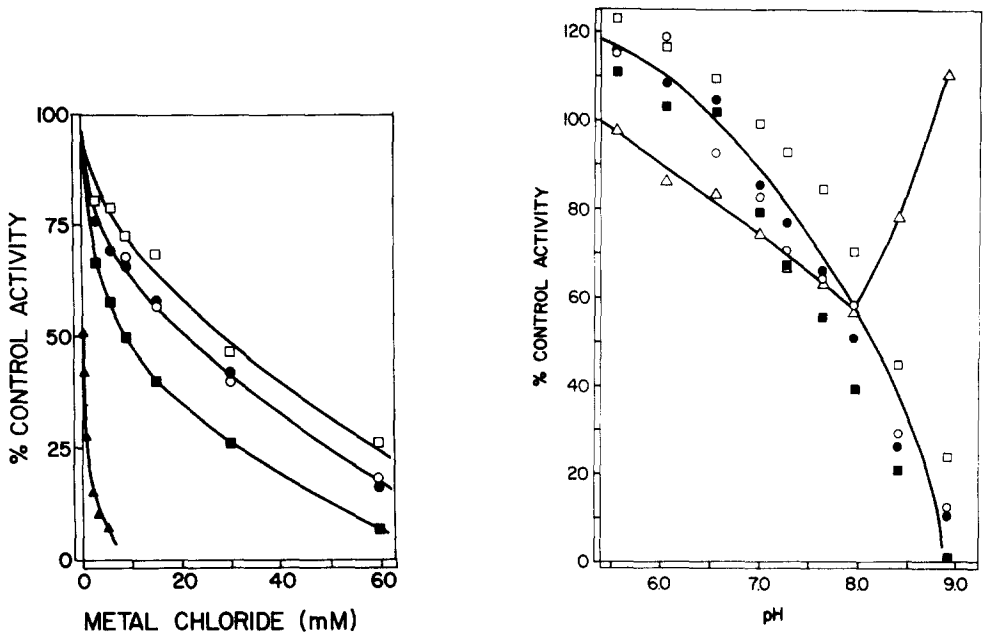


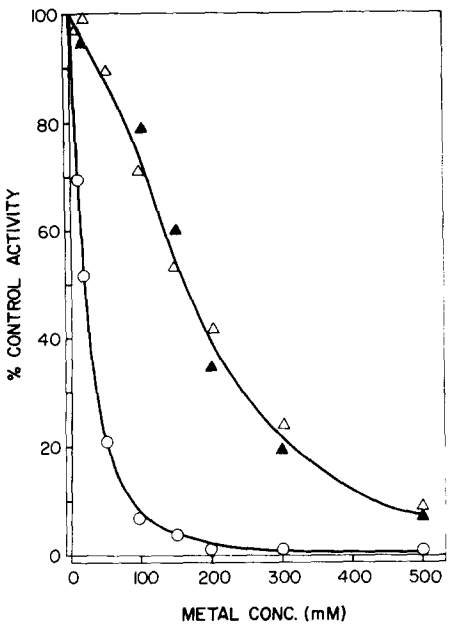
Fig. 1. Concentration dependence of cation inhibition of reverse transhydrogenase. The effect of the indicated concentrations of Ca^{2+} (○), Mg^{2+} (●), Mn^{2+} (■), Sr^{2+} (□), and La^{3+} (▲) on transhydrogenase (0.5 mg submitochondrial particle protein) was assessed at pH 7 as described under Materials and Methods. Percent control activity compares experimental rates in the presence of cation to the rate obtained in the absence of added cations, 258 nmol AcPyADH formed/min per mg protein.

Fig. 2. pH dependence of divalent cation inhibition of reverse transhydrogenase. Submitochondrial particles (0.50 mg protein) were assayed for transhydrogenase activity at the indicated pH and in the presence of either 10 mM Ca^{2+} (○), 10 mM Mg^{2+} (●), 5 mM Mn^{2+} (■), 10 mM Sr^{2+} (□), or 200 μM La^{3+} (△) as described under Materials and Methods. Control rates were in the absence of added cations.

cations also inhibit transhydrogenase, albeit at much higher concentrations (Fig. 3). About 200 mM K^+ or Na^+ was required for 50% inhibition of transhydrogenation, whereas Tl^+ was a nearly 10-fold more effective inhibitor. Thallium has also been found to be nearly 10-fold more effective than K^+ in binding to ($Na^+ + K^+$)-ATPase [17—20], Ca^{2+}-dependent ATPase [21], diol dehydrase [22], and pyruvate kinase [22,23]. Similar to divalent and trivalent cations, greater inhibition by monovalent ions was observed at higher pH. Inhibition by either 300 mM Na^+ or K^+ increased from 40% at pH 5.5 to 95% at pH 8.5.

Kinetics of cation inhibition

Rydström [24] has presented kinetic data suggesting that proton concentration regulates transhydrogenase activity by converting an inactive conformation of the enzyme to an active conformation in a manner similar to membrane energization. In view of the fact that cation inhibition is pH dependent, it was of interest to determine if cations compete with protons for a binding site or sites on the enzyme. Fig. 4 shows a double-reciprocal plot of the effect of 10 mM and 30 mM Mg^{2+} on the reduction of $AcPyAD^+$ by NADPH over the



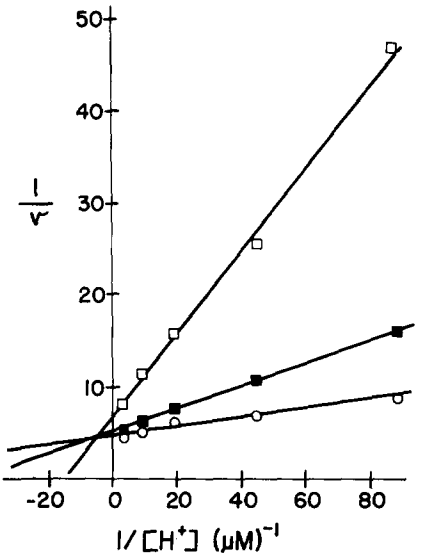


Fig. 3. Concentration dependence of monovalent cation inhibition of reverse transhydrogenase. Submitochondrial particles (0.61 mg protein) were assayed at pH 7.9 for transhydrogenase activity in the presence of the indicated concentration of K^+ (▲), Na^+ (△), or Tl^+ (○) as described under Materials and Methods. Percent control activity compares experimental rates in the presence of cation to the rate obtained in the absence of added cation.

Fig. 4. Kinetics of Mg^{2+} inhibition of reverse transhydrogenase at various proton concentrations. Submitochondrial particles (0.50 mg protein) were assayed for transhydrogenase activity in the presence of either 10 mM $MgCl_2$ (■), 30 mM $MgCl_2$ (□), or no added cations (○) over the pH range 6.5—8.0 in Tris/acetate buffer as described under Materials and Methods.

pH range 6.5—8.0. These data are not consistent with pure competition between protons and Mg^{2+} indicating either that cations (a) do not directly compete for a protonation site that regulates activity or (b) not only compete with a regulatory protonation site, but also influence activity by binding to other sites.

Previous kinetic studies using regenerating systems for NAD^+ in the reverse transhydrogenase reaction and for NADH in the forward reaction implicated Mg^{2+} as a competitive inhibitor of NAD^+ binding and a non-competitive inhibitor of NADH binding [11]. These studies could not be confirmed by Rydström, who concluded that Mg^{2+} was not competitive with either the NAD or NADP substrate [8]. A re-investigation of Mg^{2+} inhibition using substrate analogs instead of substrate regeneration systems is presented in Figs. 5 and 6. As demonstrated in Fig. 5, Mg^{2+} was competitive with NADPH (panel A) and non-competitive with $AcPyAD^+$ (panel B) in the reverse transhydrogenase reaction. Correspondingly, in the forward reaction Mg^{2+} is a competitive inhibitor of thio-$NADP^+$ (Fig. 6, panel A) and a non-competitive inhibitor of NADH (Fig. 6, panel B). In both directions the K_i for Mg^{2+} was about 2.5 mM at pH 7.0. Similar results were found with Ca^{2+} (O'Neal, S.G. and Fisher, R.R., unpublished data).

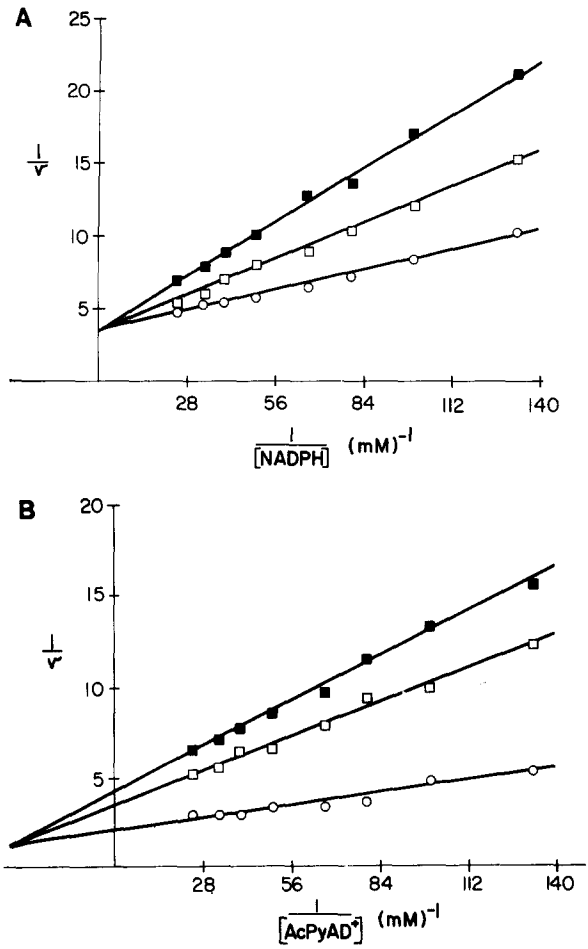


Fig. 5. Kinetics of Mg^{2+} inhibition of reverse transhydrogenase. Submitochondrial particles (0.04 mg protein) were assayed at 22°C in the absence and presence of Mg^{2+}, in a medium (3.0 ml) containing 0.1 M Tris/acetate, pH 7.0, and 1.4 μM rotenone. In Expt. A, $AcPyAD^+$ was present at 120 μM with either no Mg^{2+} (○), 2.5 mM Mg^{2+} (□), or 5 mM Mg^{2+} (■). In Expt. B, NADPH was present at 120 μM with either no Mg^{2+} (○), 5 mM Mg^{2+} (□), or 10 mM Mg^{2+} (■).

Effect of cations on Nbs_2 inactivation

We have previously reported that Mg^{2+} simulates the rate of transhydrogenase inhibition by the sulfhydryl reagent Nbs_2, presumably by increasing the reactivity of a NADP site cysteinyl residue [14,25]. Table I shows the effect of Mg^{2+} on the transhydrogenase inhibition by a variety of sulfhydryl reagents. Significant stimulation of inactivation was found with disulfides, particularly acid disulfides. Hence, it might be argued that Mg^{2+} potentiation of Nbs_2 inactivation results from a nonspecific interaction of the cation with the Nbs_2 carboxylate, thus increasing the electrophilicity of the disulfide bond. This seems unlikely since the reaction of equimolar concentrations of reduced glutathione and Nbs_2 at pH 7.4, monitored as an increase in free thionitrobenzoate anion absorbance at 412 nm, is unaffected by the presence of 1—10

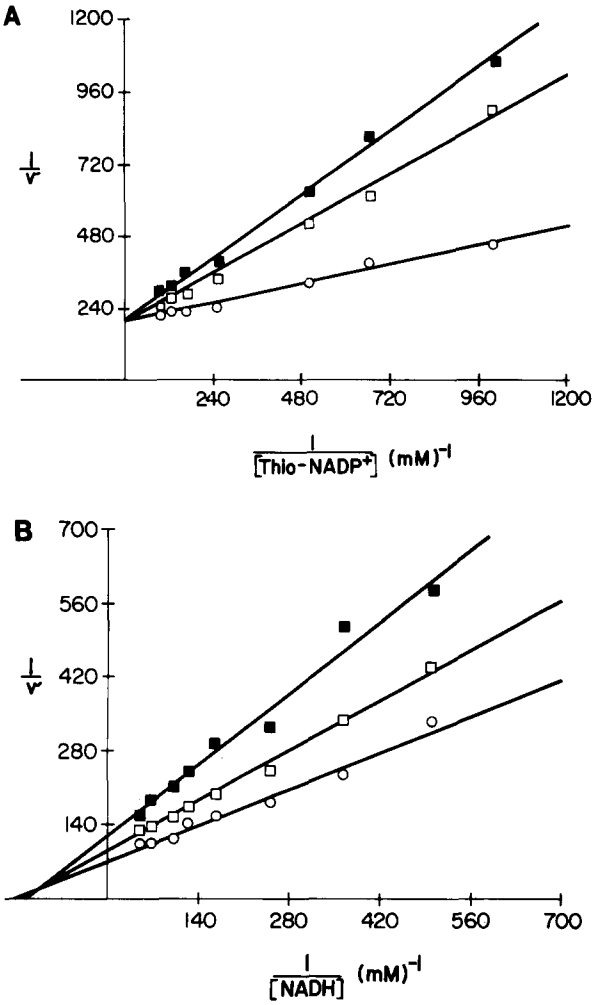


Fig. 6. Kinetics of Mg^{2+} inhibition of forward transhydrogenase. Submitochondrial particles (0.23 mg protein) were assayed at 22°C in the absence or presence of Mg^{2+}, in a medium (3.0 ml) containing 0.1 M Tris/acetate, pH 7.0, and 1.4 μM rotenone. In Expt. A, NADH was present at 120 μM with either no Mg^{2+} (○), 5 mM Mg^{2+} (□), or 10 mM Mg^{2+} (■). In Expt. B, thio-$NADP^+$ was present at 50 μM with either no Mg^{2+} (○), 7.5 mM Mg^{2+} (□), or 15 mM Mg^{2+} (■).

mM Mg^{2+}. Mg^{2+}-induced sensitivity of transhydrogenase to sulfhydryl group inactivation might also be accounted for if Mg^{2+} binding in the vicinity of the active site lowers the pK_a of reactive cysteinyl residues, thereby increasing the relative population of the more reactive mercaptide ion [26]. An investigation of the effect of Mg^{2+} on Nbs_2 inactivation at different H^+ concentrations indicates that the reactivity of the sulfhydryl group is raised slightly, not lowered (data not shown). Hence, increased sulfhydryl group reactivity may result from a Mg^{2+}-induced conformational change of the enzyme which allows greater accessibility of certain modification reagents to the NADP binding site. The lack of stimulation of *N*-ethylmaleimide inactivation by Mg^{2+} is consistent

TABLE I

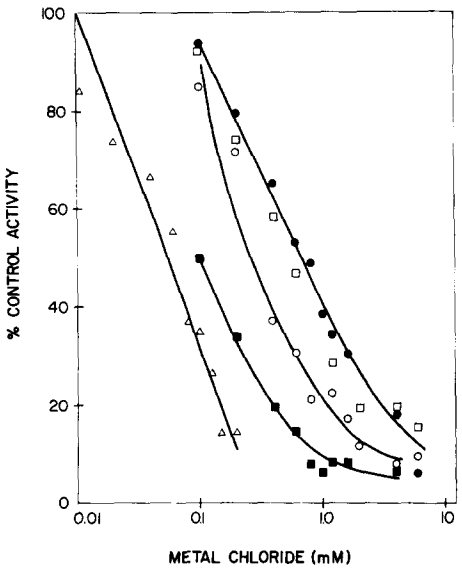
EFFECT OF $MgCl_2$ ON NON-ENERGY-LINKED TRANSHYDROGENASE INACTIVATION BY A VARIETY OF SULFHYDRYL REAGENTS

Submitochondrial particles (0.36 mg protein) were preincubated at pH 7.5 in the presence or absence of 2 mM $MgCl_2$ plus the indicated sulfhydryl reagent, and then were assayed for reverse non-energy-linked transhydrogenase activity as described under Materials and Methods. Activity indicates nmol AcPyADH formed/min per mg protein. Sulfhydryl reagents were freshly prepared by solubilizing in a minimal volume of ethanol and then diluting to volume with distilled water. NEM, *N*-ethylmaleimide.

Preincubation	Transhydrogenase activity			
	$-Mg^{2+}$		$+Mg^{2+}$	
	Activity	% control	Activity	% control
Expt. I				
None	145	100	145	100
Nbs_2 (20)	72	49	11	8
FDS (200)	83	57	62	43
DTP (200)	80	55	62	43
PCMBS (3)	109	75	93	64
TDET (500)	91	63	81	56
Expt. II				
None	125	100	124	99
Nbs_2 (20)	64	51	15	12
DTNA (100)	81	64	36	29
PCMB (4)	63	50	63	50
Expt. III				
None	115	100	114	100
Nbs_2 (20)	53	46	22	19
NEM (1000)	41	36	40	35

with the observation that the reagent inhibits transhydrogenase through modification of a peripheral cysteine and does not react with the NADP-site cysteinyl residue [25].

A comparison of the effects of Mg^{2+} and other multivalent cations on Nbs_2 inactivation of transhydrogenase is given in Fig. 7. As can be seen all the ions tested promoted inactivation, with half-maximal effects observed at 9.6 mM Mg^{2+}, 0.25 mM Ca^{2+}, 0.1 mM Mn^{2+}, 0.4 mM Sr^{2+}, and 50 μM La^{3+}. Increasing concentrations of monovalent cations in the range of 0—20 mM affect a modest stimulation of Nbs_2 inactivation (Fig. 8). However, this effect is reversed and protection against inactivation results if the cation concentrations exceed 75 mM. This suggested that both high and low-affinity cation binding sites exist on transhydrogenase which, when occupied, induce the formation of distinct transhydrogenase conformers with differing sulfhydryl group reactivities. If two such classes of cation binding sites exist, it would be expected that enhancement of Nbs_2 inactivation afforded by low concentrations of di- and trivalent cations, presumably bound to the high-affinity sites, would be reversed in the presence of high concentrations of monovalent cations binding to the low-affinity sites. Table II shows the effect of 120 mM K^+ on the di- and trivalent cation stimulation of transhydrogenase inactivation by Nbs_2. Under conditions where high K^+ concentration exerts essentially no net influence over sulfhydryl group reactivity, the stimulation of Nbs_2 inactivations by other cations is significantly decreased.



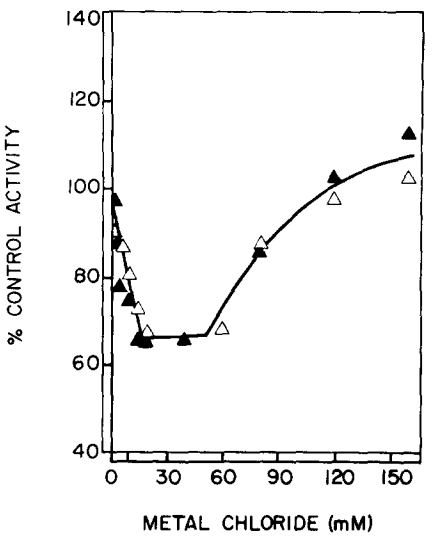


Fig. 7. Effect of various cations on Nbs_2 inactivation of transhydrogenase. Submitochondrial particles (0.45 mg protein) were preincubated (pH 7.5) for 4 min at 22°C in the presence of 20 μM Nbs_2 plus the indicated concentration of divalent cation, then assayed for transhydrogenase activity as described under Materials and Methods. Percent control activity compares experimental rates after partial Nbs_2 inactivation in the presence of each cation to the rate obtained in the absence of that cation: △, La^{3+}; ○, Ca^{2+}; □, Sr^{2+}; ●, Mg^{2+}; ■, Mn^{2+}.

Fig. 8. Effect of monovalent cations on Nbs_2 inactivation of transhydrogenase. Submitochondrial particles (0.50 mg protein) were preincubated (pH 7.5) for 4 min at 22°C in the presence of 20 μM Nbs_2 and the indicated concentration of cation, K^+ (▲) or Na^+ (△), then assayed for transhydrogenase activity all as described under Materials and Methods. Percent control activity compares experimental rates after partial Nbs_2 inactivation in the presence of each cation to the rates obtained in the absence of that cation.

TABLE II

EFFECT OF 120 mM KCl ON DI- AND TRIVALENT CATION POTENTIATION OF Nbs_2 INACTIVATION OF NON-ENERGY-LINKED TRANSHYDROGENASE

Submitochondrial particles (0.3 and 0.55 mg protein for Expts. I and II, respectively) were preincubated at 22°C for 4 min in a medium (pH 7.5) containing 20 μM Nbs_2 plus 120 mM KCl and other cations as indicated, and were then assayed for transhydrogenase activity as described under Materials and Methods. In each experiment percent control activity has been computed by considering the activity obtained in the absence of Nbs_2 and cations as 100%. Control rates were 170 and 95 nmol. AcPyADH formed/min per mg protein for Expts. I and II, respectively.

Precincubation additions (μM)	% control activity	
	— KCl	+ KCl
Expt. I		
None	59	62
Ca^{2+} (250)	31	66
Sr^{2+} (400)	25	44
Mg^{2+} (600)	25	44
Mn^{2+} (100)	34	44
Expt. II		
None	65	68
La^{3+} (50)	46	62

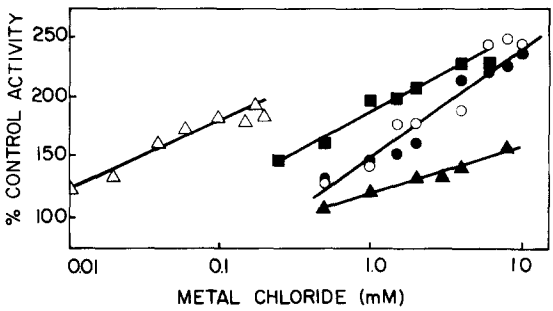


Fig. 9. Effect of divalent cations on transhydrogenase thermostability. Submitochondrial particles (0.36 mg protein) were preincubated at 53°C for 110 s in the presence of the indicated cation concentration, then were assayed for transhydrogenase activity as described under Materials and Methods. Percent control activity compares experimental rates following incomplete thermal inactivation in the presence of each cation to the rate obtained in the absence of that cation: △, La^{3+}; ▲, K^+; ○, Ca^{2+}; ●, Mg^{2+}; ■, Mn^{2+}.

Effect of cations on thermal inactivation

Several divalent cations stabilize rat liver mitochondrial transhydrogenase against thermal inactivation [12]. Thermostability studies have shown that Mg^{2+} enhances transhydrogenase stability and that the bovine heart enzyme exists in three different conformers: the native enzyme, the $NADP^+$-enzyme complex, and the NADPH-enzyme complex [14]. The effects of several cations on the thermostability of bovine heart transhydrogenase are portrayed in Fig. 9. Cation transhydrogenase inhibitors prevent thermal inactivation, with half-maximal protection afforded at about 1—2 mM Mg^{2+}, 2 mM Ca^{2+}, 0.5 mM Mn^{2+} and 25 μM La^{3+}. Maximal protection represents 70—85% of the activity of untreated control particles. K^+ was far less effective in providing protection in the same concentration range as the divalent cations, but greater than 95% protection was seen at 200 mM K^+, with half-maximal protection at about 20 mM.

Effect of cations on proteolytic inactivation

It is apparent that cation interaction alters the conformation of rat liver submitochondrial particle transhydrogenase, since 1 mM of Mg^{2+}, Ca^{2+}, Mn^{2+}, Sr^{2+}, or Ba^{2+} promote the tryptic inactivation of the enzyme [12]. In contrast to the rat liver enzyme, cations prevent trypsinolysis of the bovine heart enzyme (Fig. 10). Optimal protection against trypsin is nearly complete with Mn^{2+} (greater than 95%), whereas Ca^{2+} (89%) and Mg^{2+} (79%) are somewhat less effective. As was observed in the thermal and Nbs_2 inactivation experiments above, the concentration of divalent cations effecting half-maximal protection was in the range of 1—2 mM (Fig. 10). In the range 1—10 mM, K^+ provided only minimal protection, but half-maximal and total protection were seen at 20 mM and 200 mM K^+, respectively.

Effect of membrane energization on Mg^{2+}-dependent properties

Energy-linked transhydrogenation is inhibited to a much lesser degree by Mg^{2+} than is the non-energy-linked reaction [11,13]. Rydström and coworkers [9,27] proposed that membrane energization converts transhydrogenase from

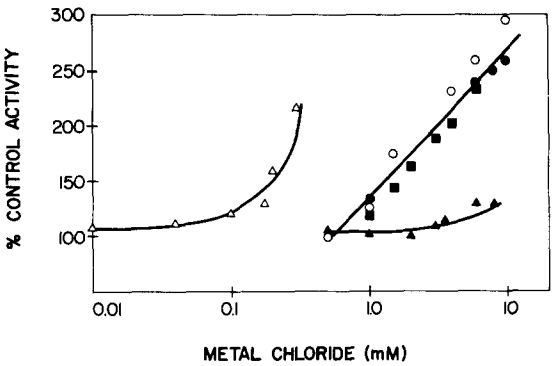


Fig. 10. Effect of divalent cations on tryptic inactivation of transhydrogenase. Submitochondrial particles (0.36 mg protein) were preincubated at 22°C for 2 min in the presence of 11 μg trypsin and the indicated cation concentration, the inactivation terminated, and then the particles assayed all as described under Materials and Methods. Percent control activity compares experimental rates after partial proteolytic inactivation in the presence of each cation to the rates obtained in the absence of that cation: △, La^{3+}; ▲, K^+; ○, Ca^{2+}; ●, Mg^{2+}; ■, Mn^{2+}.

an 'inactive' to an 'active' conformation. The primary evidence presented for an energy-linked change in transhydrogenase conformation was alterations in substrate Michaelis constants, which for NADH increased from 9 μM to 12.6 μM, and more impressively, for $NADP^+$ decreased from 40 μM to 6.5 μM [27]. A simple explanation for the lower effectiveness of Mg^{2+} inhibition could be provided if the 'energized' transhydrogenase conformation has a much lower affinity for Mg^{2+} than the 'non-energized' conformation. To test this, proteolytic and Nbs_2 inactivation were used as probes to detect Mg^{2+} binding to transhydrogenase under non-energized and energized conditions. It was expected that if energization decreases the enzyme affinity for Mg^{2+}, then the magnitude

TABLE III

EFFECT OF MEMBRANE ENERGIZATION ON Mg^{2+} PROTECTION AGAINST PROTEOLYSIS OF TRANSHYDROGENASE

Submitochondrial particles (0.38 mg protein) were preincubated for 1 min at 22°C in a medium consisting of 20 mM Tris/acetate, pH 7.5, 7.5 mM succinate, 3 μg oligomycin, 30 μg trypsin, and also 100 μg trypsin inhibitor and 5 mM $MgCl_2$ where indicated, then assayed for non-energy-linked transhydrogenase activity as described under Materials and Methods except that 0.22 μg antimycin A was added to the assay mixture to prevent succinate oxidation, permitting the assay of transhydrogenase under non-energized conditions. For non-energized preincubation conditions, antimycin A was present in the preincubation mixture.

Additions	Transhydrogenase rate (nmol AcPyADH/min per mg protein)	% control
Non-energized particles		
Trypsin + trypsin inhibitor	252	100
Trypsin	55	22
Trypsin + $MgCl_2$	163	64
Energized particles		
Trypsin + trypsin inhibitor	252	100
Trypsin	69	27
Trypsin + $MgCl_2$	160	63

TABLE IV

EFFECT OF MEMBRANE ENERGIZATION ON Mg^{2+} ENHANCEMENT OF Nbs_2 INACTIVATION OF TRANSHYDROGENASE

Submitochondrial particles (0.38 mg protein) were preincubated for 1 min at 22°C in a medium consisting of 20 mM Tris/acetate, pH 7.5, 7.5 mM succinate, 3 μg oligomycin, 20 μM Nbs_2, and 2 mM $MgCl_2$ where indicated, then assayed for non-energy-linked transhydrogenase activity as described in Table III.

Additions	Transhydrogenase rate (nmol AcPyADH/min per mg protein)	% control
Non-energized particles		
None	244	100
Nbs_2	127	52
$MgCl_2$	247	100
Nbs_2 + $MgCl_2$	80	32
Energized particles		
None	236	100
Nbs_2	115	49
$MgCl_2$	235	100
Nbs_2 + $MgCl_2$	80	34

of the effects of the cation on proteolysis and Nbs_2 inactivation would be similarly decreased. Table III shows that tryptic inactivation of submitochondrial transhydrogenase is inhibited to a similar extent by Mg^{2+} in non-respiring submitochondrial particles and those oxidizing succinate. Furthermore, in Table IV it is shown that concomitant respiration has little affect on Mg^{2+} enhancement of Nbs_2 inactivation.

Discussion

It is apparent from the data presented that Mg^{2+} inhibits transhydrogenase by binding in the NADP domain of the active site in a manner competitive with the substrate. No evidence was found for inhibitory Mg^{2+} binding at the NAD domain or elsewhere on the enzyme. Energization of transhydrogenase by respiration apparently does not alter the affinity of the enzyme for Mg^{2+} as measured by the effect of the ion on inactivation by proteolysis and Nbs_2. Hence, this is an unlikely explanation for the lower susceptibility of energy-linked transhydrogenation to inhibition by Mg^{2+} [11,13]. An alternative explanation is provided by the work of Rydström and coworkers [9,27], who demonstrated that the affinity of transhydrogenase for $NADP^+$ is increased by nearly 6-fold on energization. This would necessarily decrease the effectiveness of Mg^{2+} as a competitive inhibitor.

Several cations other than Mg^{2+} were bovine heart transhydrogenase inhibitors. The order of inhibitory effectiveness was $La^{3+} > Mn^{2+} > Ca^{2+} \approx Mg^{2+} > Sr^{2+} > Na^+ \approx K^+$. Although a detailed kinetic analysis on cations other than Mg^{2+} and Ca^{2+} has not been performed, the results suggest a common inhibitory mechanism for all cations. For instance, each cation was more effective with increasing pH in the range of 5.5—9.0. Whereas divalent cations slightly stimulated transhydrogenation at low pH, monovalent cations at 300 mM were inhibitory at all pH values, possibly because of nonspecific ion

effects. The K_i for Mg^{2+} is about 2.5 mM. Significantly, the Mg^{2+} concentrations giving half-maximal stimulation of Nbs_2 inactivation, protection against thermal inactivation, and protection against tryptic inactivation were about 0.6 mM, 1.5 mM, and 2 mM, respectively. This suggests that Mg^{2+} binding in the active site alters the transhydrogenase to a more thermostable conformation having one or more arginyl or lysyl residues less susceptable to cleavage by trypsin, and a sulfhydryl group that is more accessible to Nbs_2 than the native conformation. This notion is complemented by the observation that the other cation inhibitors also mimic Mg^{2+} in these properties, giving the same general trend of effectiveness as in inhibition, i.e. trivalent > alkaline earth > monovalent cations. This order for the strength of cation binding to transhydrogenase, also observed in several other proteins including bovine serum albumin, lysozyme, myoglobin, and ovalbumin [28], is described by the Irving-Williams order for the stability of metal-ligand complexes [29]. The adherence of transhydrogenase inhibition to the Irving-Williams order suggests that carboxylates or amines may comprise the inhibitory cation binding site.

The role of mitochondrial pyridine dinucleotide transhydrogenase in heart muscle metabolism is unclear [30]. However, certain aspects of the interaction between heart mitochondria and cations are being elucidated. Heart mitochondria actively accumulate both Mg^{2+} and Ca^{2+} in an energy-dependent and apparently competitive manner [33]. The matrix enzymes pyruvate dehydrogenase phosphatase [31] and isocitrate dehydrogenase (NAD) [32] are known to be regulated, in part, by Ca^{2+}. Furthermore, Meli and Bygrave showed that pyruvate kinase activity can be regulated in vitro through mitochondrial control of calcium influx and efflux [34]. Since cations have now been shown to be competitive inhibitors of NADP binding to transhydrogenase, it is proposed that the activity of transhydrogenase, and by extension its physiological role, may be regulated in vivo by cyclic changes in cation activity within heart muscle mitochondria.

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References

1 Danielson, L. and Ernster, L. (1963) Biochem. Biophys. Res. Commun. 10, 91—96
2 Van Dam, K. and Ter Welle, H.F. (1965) Regul, Metab. Processes Mitochondria, Proc. Symp., 7, 235—246
3 Rydström, J. (1977) Biochim. Biophys. Acta 463, 155—184
4 Lee, C.-P. and Ernster, L. (1964) Biochim. Biophys. Acta 81, 187—190
5 Höjeberg, B. and Rydström, J. (1977) Biochem. Biophys. Res. Commun. 78, 1183—1190
6 Earle, S.R., Anderson, W.M. and Fisher, R.R. (1978) FEBS Lett. 91, 21—24
7 Lee, C.-P., Simard-Duquesne, N. and Ernster, L. (1965) Biochim. Biophys. Acta 105, 397—409
8 Rydström, J. (1972) Eur. J. Biochem. 31, 496—504
9 Teixeira da Cruz, A., Rydström, J. and Ernster, L. (1971) Eur. J. Biochem. 23, 203—211
10 Andreoli, T.E., Pharo, R.L. and Sanadi, D.R. (1964) Biochim. Biophys. Acta 90, 16—23
11 Hommes, A. (1963) in Energy-Linked Functions of Mitochondria (Chance, B., ed.), pp. 39—48, Academic Press, New York
12 Blazyk, J.F., Lam, D. and Fisher, R.R. (1976) Biochemistry 15, 2843—2848
13 Rydström, J., Teixeira da Cruz, A. and Ernster, L. (1970) Eur. J. Biochem. 17, 56—66
14 O'Neal, S.G. and Fisher, R.R. (1977) J. Biol. Chem. 252, 4552—4556

15 Fisher, R.R. and Kaplan, N.O. (1973) Biochemistry 12, 1182—1188
16 Kaplan, N.O. and Ciotti, M.M. (1954) J. Am. Chem. Soc. 76, 1713—1714
17 Britten, J.S. and Blank, M. (1968) Biochim. Biophys. Acta 159, 160—166
18 Inturrisi, C.E. (1969) Biochim. Biophys. Acta 173, 567—569
19 Inturrisi, C.E. (1969) Biochim. Biophys. Acta 178, 630—633
20 Skulskii, I.A., Manninen, V. and Järneflt, J. (1973) Biochim. Biophys. Acta 298, 702—709
21 Duggan, P.F. (1977) J. Biol. Chem. 252, 1620—1627
22 Williams, R.J.P. (1970) Chem. Soc. Q. Rev. 24, 331—365
23 Kayne, F.J. (1971) Arch. Biochem. Biophys. 143, 232—239
24 Rydström, J. (1974) Eur. J. Biochem. 45, 67—76
25 Earle, S.R., O'Neal, S.G. and Fisher, R.R. (1978) Biochemistry 17, 4683—4690
26 Polgar, L. (1974) FEBS Lett. 38, 187—190
27 Rydström, J., Teixeira da Cruz, A. and Ernster, L. (1971) Eur. J. Biochem. 23, 212—219
28 Katz, S. and Roberson, L.C. (1976) Bioinorg. Chem. 6, 143—154
29 Irving, H. and Williams, R.J.P. (1953) J. Chem. Soc. 1953, 3192—3210
30 Rydström, J., Hoek, J.B. and Ernster, L. (1976) The Enzymes 13, 51—88
31 Pettit, F.H., Roche, T.E. and Reed, L.J. (1972) Biochem. Biophys. Res. Commun. 49, 563—571
32 Vaughan, H. and Newsholme, E.A. (1969) FEBS Lett. 5, 124—126
33 Jacobus, W.E., Tiozzo, R., Lugli, G., Lehninger, A.L. and Carafoli, E. (1975) J. Biol. Chem. 250, 7863—7870
34 Meli, J. and Bygrave, F.L. (1972) Biochem. J. 128, 415—420