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Rapid Report

Activation and inhibition of mitochondrial transhydrogenase by metal ions

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Mitochondrial transhydrogenase has been reported previously to be inhibited by high, rather non-physiological concentrations (in the range of 2–20 mM) of divalent cations. We show that the enzyme could be activated by low (from about 1 μM to 1 mM) concentrations of Ca^{2+} and Mg^{2+}, which are within physiological range. These results bring in line the effects observed with mitochondrial enzyme to the findings with bacterial transhydrogenases. The activation of transhydrogenase by divalent cations is interpreted as an increase in affinity of the NADP(H)-binding site of the enzyme-NAD(H) complex. Reported effects of the metal ions could be important for the enzyme function in vivo.

Mitochondrial pyridine dinucleotide transhydrogenase (H^+-thase) couples the transfer of hydride ion equivalents between NAD(H) and NADP(H) to the translocation of protons across the inner membrane [1,2]:

$$NADH + NADP^+ + H^+_{out/cytosol} \rightleftharpoons NADPH + NAD^+ + H^+_{in/matrix} \quad (1)$$

Energization of submitochondrial particles enhances the 'forward' reaction (from left to right, Eqn. 1) several fold [1,2]. There are separate binding domains on the enzyme for NAD(H) and NADP(H) [1,3,4]. The physiological role of mitochondrial H^+-thase is still a matter of controversy [1,2,5]. One possible function is that H^+-thase is involved in the regulation of the mitochondrial NAD(P)H levels and transmembrane proton electrochemical gradient, Δp [5,6].

Dehydrogenases of the tricarboxylic acid cycle are activated by micromolar concentrations of Ca^{2+}, and this has important consequences for the respiratory activity of mitochondria [7]. It is therefore of interest to establish whether H^+-thase is regulated by Ca^{2+} and other metal ions.

Divalent metal ions, including Mg^{2+} and Ca^{2+}, previously have been shown to inhibit non-energy-linked forward and reverse transhydrogenation, with a lesser effect on the energy-linked reaction [8–12]. Inhibition was observed only in the presence of high concentrations of cations – in the range of 2–20 mM with K_i values of about 3–5 mM. These concentrations are much higher than those in vivo – a recent estimate of the free $[Mg^{2+}]$ in mitochondria is about 0.3 mM, while the free $[Ca^{2+}]$ is about 1 μM [7]. Therefore, it is unlikely that such inhibition has a physiological role. Monovalent cations (K^+, Na^+) inhibited H^+-thase at very high concentrations with a K_i of about 150 mM [12].

Bacterial membrane-bound transhydrogenases are essentially analogous to the mitochondrial enzyme [1], but, in contrast, they have been shown to be activated by submillimolar concentrations of Ca^{2+} and Mg^{2+} in *Rhodobacter capsulatus* [13] and *Escherichia coli* [14]. In view of the apparent difference in the action of metal ions on the mitochondrial and bacterial enzymes we decided to study the effects in more detail.

Previous experiments on the effects of metal ions on mitochondrial H^+-thase were conducted in the absence of chelators [8–12], which may have resulted in a background presence of cations, especially Ca^{2+}, in the medium. We used EDTA-containing buffer at low ionic strength (10 mM Tricine) to avoid such distortions. Under these conditions we were able to find significant activation of mitochondrial H^+-thase by low concentrations of divalent cations (within the physiological range).

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Beef heart mitochondria and submitochondrial particles (SMPs) were prepared essentially as described [15]. SMPs were suspended in 0.25 M sucrose, 10 mM Hepes (pH 7.6) and kept frozen at −20°C until use. H^+-thase activity was assayed at 30°C in a medium containing 0.25 M sucrose, 10 mM Tricine-NaOH (pH 7.6), 0.2 mM EDTA, SMPs (0.25–0.5 mg protein), 2 μM rotenone, 1 μg/ml oligomycin and the indicated concentrations of nucleotides in a total volume of 3 ml. Assays for non-energy-linked forward and reverse reactions also contained 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The energy-linked forward reaction was driven by 2.5 mM succinate in air-saturated medium. Reaction rates were determined by monitoring the increase in absorbance of the reduced forms of the $NADP^+$ and NAD^+ analogs, thio-$NADP^+$ (at 398–470 nm, assuming a millimolar extinction coefficient of 11.3 cm^{-1} [12]) and 3-acetylpyridine adenine dinucleotide, $AcPyAD^+$ (at 375–455 nm, extinction coefficient 6.1 cm^{-1} [16]). Control rates were obtained in the absence of added metals. Indicated concentrations of cations represent calculated estimations of free concentrations based on known stability constants of metals in EDTA buffer at pH 7.6 [17]. Metal ions were added as stock solutions of $CaCl_2$, $MgSO_4$ or KCl.

The effects of divalent cations on H^+-thase reactions in the presence of nearly saturating concentrations of substrates are presented in Fig. 1. Both the non-energy-linked forward and the reverse reactions (Fig. 1B and C) were activated by low (approx. 1 μM) concentrations of Ca^{2+} or Mg^{2+} with maximal activation at about 0.1 mM and inhibition at higher concentrations, up to 20 mM. Inhibition by Ca^{2+} only returned the rates to the control, unactivated level, whereas Mg^{2+} inhibited both reactions down to 20% of the control level with a K_i of about 5–7 mM. The forward energy-linked reaction (Fig. 1A) was activated by low concentrations of cations but higher concentrations did not lead to inhibition. Interpretation of the data on the energy-linked reaction is complicated, since there might be some effects of cations on the proton permeability of SMP membranes or on the rate of respiration (we observed about 10% stimulation of respiration by cations under similar conditions, not shown). However, the effects on the non-energy-linked reactions, when SMPs are uncoupled by FCCP, should be free from such distortions.

KCl produced similar activation-inhibition dependences, but at higher concentrations, with maximal activation of the non-energy-linked reactions at about 30 mM (Fig. 2). The ionic stregth of this solution is much higher than that of 0.1 mM divalent cation, suggesting that effects are somewhat ion-specific. This specificity is indicated also by the difference in inhibition by Ca^{2+} and Mg^{2+}, mentioned above. However, the effects of

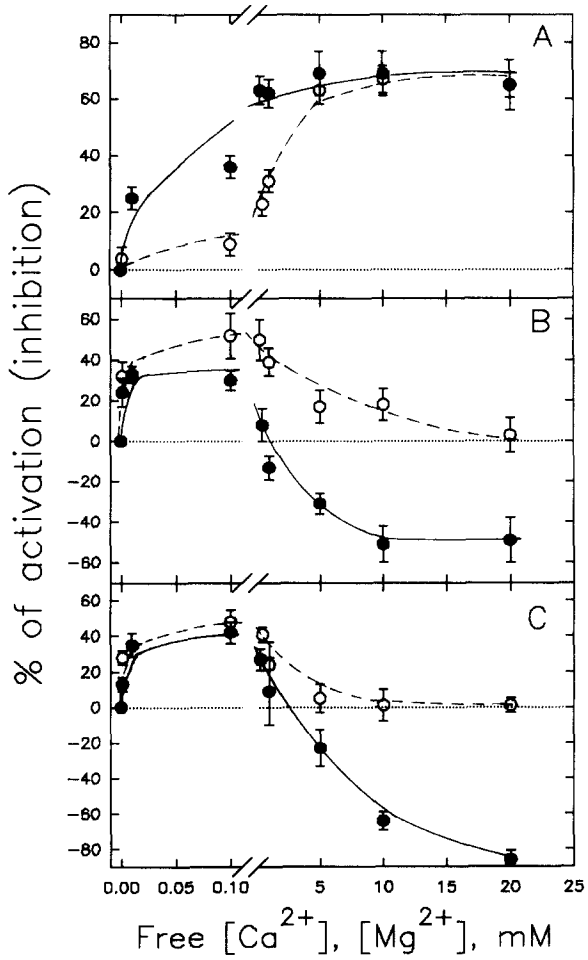

Fig. 1. Effects of divalent cations on H^+-thase. (A) Forward energy-linked reaction (NADH → thio-$NADP^+$ in the presence of succinate). (B) Forward non-energy-linked reaction (NADH → thio-$NADP^+$). (C) Reverse reaction (NADPH → $AcPyAD^+$). •, Mg^{2+}, ○, Ca^{2+}. The concentrations of substrates were: 200 μM NADH and 100 μM thio-$NADP^+$ (A, B), 200 μM NADPH and 200 μM $AcPyAD^+$ (C). The conditions for H^+-thase assays are described in text. Control rates (0% activation), obtained in absence of added cations, were about 40, 7 and 100 nmol/min per mg protein, respectively, for forward energy-linked, forward non-energy-linked and reverse reactions. Points represent mean ±S.E. from three experiments.

different metal ions were not additive, that is, there was no activation by divalent cations in presence of 30 mM KCl (not shown).

Thus, we can conclude that the previously observed inhibition of H^+-thase by metal ions represents only part of the dependence, because in the absence of chelators small concentration of ions (but enough to activate the enzyme) might be present in the medium. Non-additivity of the effects of K^+ and divalent cations may raise the question whether the activation by Ca^{2+} or Mg^{2+} takes place in vivo, where a high concentration of K^+ is likely to be present. However, the total concentration of enzymes inside mitochondrial matrix is very high [18], and therefore the local concentrations

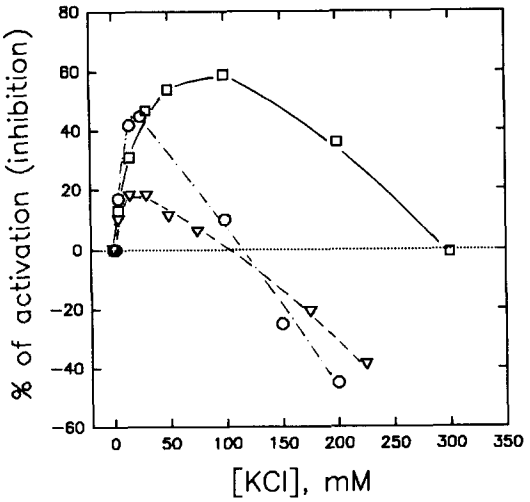

Fig. 2. Effects of KCl on H^+-thase. □, forward energy-linked reaction; ▽, forward non-energy-linked reaction; ○, reverse reaction. The conditions were as described in Fig. 1.

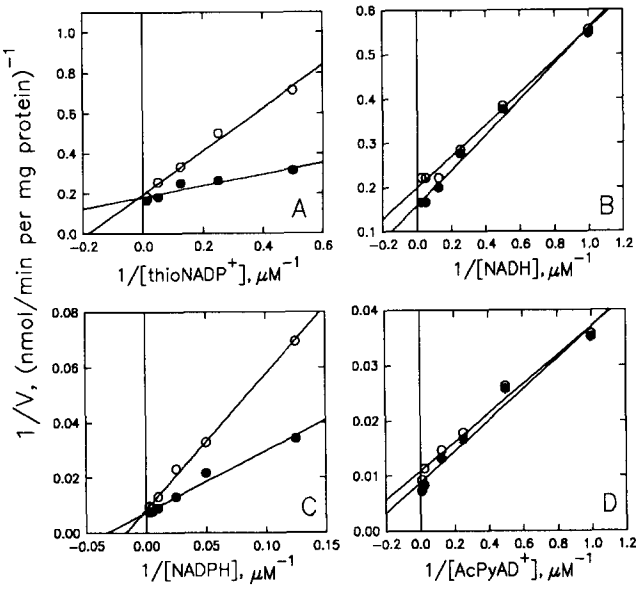

Fig. 3. Kinetics of Mg^{2+} activation of H^+-thase. (A, B) Forward non-energy-linked reaction; (C, D) reverse reaction. ○, control rates (no added cation); ●, in the presence of 0.1 mM Mg^{2+}. The concentrations of complementary substrates were: (A) 100 μM NADH; (B) 100 μM thio-$NADP^+$; (C) 100 μM $AcPyAD^+$; (D) 200 μM NADPH. Assay conditions as described in text.

of metal ions could, in principle, be substantially lower than bulk concentrations.

In order to establish the nature of the activation, steady-state kinetics in the presence and absence of divalent cations were analysed. Fig. 3 shows that activation by 0.1 mM Mg^{2+} was competitive (or compulsory) with respect to thio-$NADP^+$ and NADPH (graphs A, C) and approximately uncompetitive with respect to $AcPyAD^+$ and NADH (graphs B, D) in the presence of a saturating concentration of the complementary substrate (for description of terms, see Ref. 19). The results were essentially similar for both the forward non-energy-linked (A, B) and the reverse (C, D) reaction. Similar dependences were obtained with 0.1 mM Ca^{2+} (not shown). The conclusions of the graphical plots were confirmed by direct fitting of the data (by non-linear regression) to the Michaelis-Menten equation (Table I). With respect to the NADP substrates, activation by 0.1 mM of either Mg^{2+} or Ca^{2+} led to a significant decrease in apparent K_m (between 2- and 4-fold) with a small increase in V_{max}. However, with respect to the NAD substrates, the same additions led to approximately similar increase both in K_m and V_{max}, in agreement with the proposed uncompetitive mechanism.

Thus, from Fig. 3 and Table I it follows that divalent cations in low concentrations affect the enzyme-NAD(H) complex (uncompetitive mechanism), but act on the free enzyme with respect to NADP substrates (competitive mechanism). From this we can conclude that cations increase the affinity of H^+-thase for NADP(H) and analogues, but only when NAD(H) is bound to its own site. Presumably binding of a metal ion can change the conformation of the NADP(H)-binding site of the enzyme-NAD(H) complex (but not of the free enzyme) to one more favorable for NADP(H) binding. It is not clear where on the enzyme

TABLE I

Kinetic parameters of the forward non-energy-linked and reverse reaction of H^+-thase.

Experiments were performed in the absence of added cations and in the presence of the indicated free concentrations of divalent cations. Concentrations of the indicated H^+-thase substrates were varied in the presence of nearly saturating concentrations of complementary substrates: 100 μM NADH, 100 μM thio-$NADP^+$, 100 μM $AcPyAD^+$ and 200 μM NADPH, respectively. Assay conditions as described in text. Apparent K_m (in μM) and V_{max} (in nmol/min per mg protein) values were estimated using non-linear regression

	Forward				Reverse			
	Thio-$NADP^+$		NADH		NADPH		$AcPyAD^+$	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Control	7.1	5.7	1.6	4.9	69	130	3.5	104
+0.1 mM Mg^{2+}	1.6	5.7	3.1	6.7	38	151	5.6	140
+0.1 mM Ca^{2+}	3.2	6.5	2.6	6.8	36	161	7.7	153
+10 mM Mg^{2+}	2.2	2.3	3.2	3.3	69	37	18	35

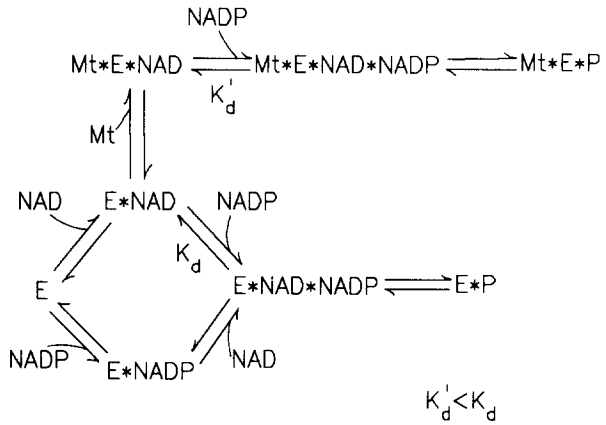

Fig. 4. Proposed mechanism of the activation of H^+-thase by low concentrations of divalent cations (Mt). E, H^+-thase, NAD and NADP represent respective substrates (NAD(H) and NADP(H)); P, products (substrates with exchanged H^- equivalent). K_d, dissociation constant of E-NAD-NADP complex for NADP substrate; K'_d, the same in the presence of cations.

the metal ion binds, but probably it should be close to the NADP(H)-binding site.

Taking into account the fact that H^+-thase operates through a ternary complex with random addition of substrates [1–4], the model to emerge is shown in Fig. 4. As our findings indicate, the activation by divalent cations decreases the dissociation constant of the enzyme-NAD(H)-NADP(H) complex for NADP(H) ($K'_d < K_d$), but has little effect on other rate constants.

The decrease in K_m for NADP substrates upon activation means that effects of cations are especially significant (giving up to 4-fold increase in reaction rates) at low concentrations of these nucleotides, which is likely to be the situation in vivo. Especially low could be the $NADP^+$ concentration, since the NADP(H) pool in mitochondria is highly reduced (the NADPH/$NADP^+$ ratio might be more than 100) [5].

The inhibition of H^+-thase by high concentration (10 mM) of Mg^{2+} was mixed in all cases (forward and reverse reactions, NAD and NADP substrates, Table I). This is not consistent with the results of O'Neal et al. [12], who found that inhibition by Mg^{2+} was competitive with NADP substrates and non-competitive with NAD substrates, and who concluded that inhibition was exerted on the NADP(H)-binding site. The origin of the discrepancy may lie in the fact that control rates in the experiments of O'Neal et al. were obtained in the absence of chelators, as pointed out above, and thus may characterize the enzyme under the influence of low concentration of ions. Furthermore, O'Neal et al. obtained, in fact, not purely non-competitive, but mixed inhibition with NAD substrates (Ref. 12, Figs. 5B and 6B). Our observations suggest that cations at high concentrations may have inhibitory effects on more than one of the enzyme-substrate intermediates (particularly on the release of products from the enzyme-NAD(H)-NADP(H) complex, since V_{max} is decreased in all cases, Table I), and therefore affect also parameters other than the K_d(NADP) value as in Fig. 4.

In conclusion, mitochondrial H^+-thase can be activated by low concentrations of divalent cations (from about 1 μM up to 1 mM). In contrast to previously observed inhibition, which is exerted by high, rather non-physiological levels of cations (2–20 mM), these concentrations lie within the physiological range for Ca^{2+} and Mg^{2+}. The reported results reconcile the effects observed with mitochondrial enzyme to those with bacterial transhydrogenases. The activation of mitochondrial H^+-thase by divalent cations might represent an increase in affinity of the NADP(H)-binding site of the enzyme for its substrate in the presence of bound NAD(H). These effects of metal ions could be important for the enzyme function in vivo.

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