Ca^{2+}-DEPENDENT ALLOSTERIC REGULATION OF NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENASE FROM PSEUDOMONAS AERUGINOSA

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SUMMARY

Nicotinamide nucleotide transhydrogenase from Pseudomonas aeruginosa exhibits allosteric properties and has been shown to be regulated by the prevailing [NADPH]/[$NADP^+$] ratio or by 2′-AMP. The present data obtained with membrane fragments from P. aerug. show that Ca^{2+} strongly influences the concentration of 2′-AMP or NADPH required for half-maximal stimulation. Saturating concentrations of Ca^{2+} cause full activation of the enzyme; Mn^{2+}, Mg^{2+} and K^+ are considerably less efficient and antagonistic to Ca^{2+}. Some implications of these findings for the regulatory mechanism and possible physiological function of the enzyme are considered.

Nicotinamide nucleotide transhydrogenase catalyzes the reaction

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

The enzyme isolated from Pseudomonas aeruginosa has been investigated extensively with respect to its kinetic and physical properties by Kaplan and coworkers (1-8). These authors conclude that the P. aeruginosa transhydrogenase is a flavoprotein, allosterically regulated by 2′-AMP and NADPH. Similar properties were reported for the Azotobacter vinelandii transhydrogenase (9-15). The reduction of NAD^+ by NADPH (the reverse reaction) proceeds readily in the absence of 2′-AMP, whereas the reduction of $NADP^+$ by NADH (the forward reaction) is stimulated by 2′-AMP or low concentrations of NADPH; $NADP^+$ acts as a potent inhibitor. The existence of a regulatory site specific for NADP(H) and

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2'-AMP has been considered but not proven. As shown in this paper the activation by both 2'-AMP and NADPH was strongly increased by Ca^{2+}; Mn^{2+}, Mg^{2+} and K^{+} were considerably less potent and antagonistic to Ca^{2+}. In the absence of activating nucleotides, maximal activity was obtained by saturating concentrations of Ca^{2+}. The substrate- and substrate analogue-dependent activation showed a marked specificity for NADPH, although most 2' (and 3') adenosine phosphate derivatives were active with varying efficiencies. Previously reported inhibitory effects of $NADP^{+}$ (1,6) were found to be relieved by saturating concentrations of Ca^{2+}. The implications of these findings for the regulatory mechanism of the transhydrogenase and its possible physiological function are discussed.

MATERIALS AND METHODS

Pseudomonas aeruginosa were grown on a minimal medium with succinate as the sole carbon and energy source, as described for E. coli by Sweetman and Griffiths (16). The bacterial particles were prepared essentially according to these authors, except that the final pellet was suspended in 0.15 M KCl. The particles were stored at -15°C in 1 mM dithioerythritol. The protein concentration of the suspension was adjusted to 10-15 mg/ml. The assay for the reduction of $NADP^{+}$ by NADH and for the reduction of NAD^{+} by NADPH was carried out according to Teixeira da Cruz et al. (17), except that the particles were preincubated for five minutes with 1 mM Na_2S as the sole respiratory inhibitor; the buffer of the medium was exchanged for 50 mM Tris-HCl pH 8.0. Occasionally, transhydrogenase partially purified according to Cohen and Kaplan (6) was used. All chemicals were purchased from Sigma Chem. Co. (St. Louis, U.S.A.) or Boehringer Mannheim GmbH (Mannheim, Germany).

RESULTS

The plot in Fig. 1 shows the effects of Ca^{2+} and 2'-AMP on the forward reaction. Clearly, at saturating concentrations, both Ca^{2+} and 2'-AMP affect the activity of the transhydrogenase in a sigmoidal fashion. At nonsaturating concentrations of 2'-AMP the activation was strongly Ca^{2+} dependent (Fig. 2), half-maximal activity being obtained at a concentration of 2'-

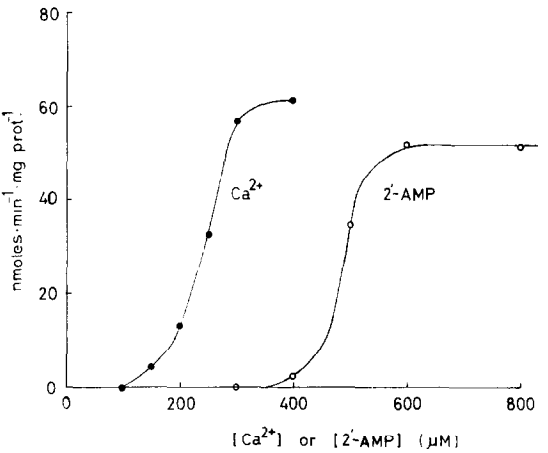

Fig. 1. Activation of the forward reaction by Ca^{2+} and 2´-AMP. The concentrations of the substrates were: 100 μM NADH and 100 μM $NADP^+$.

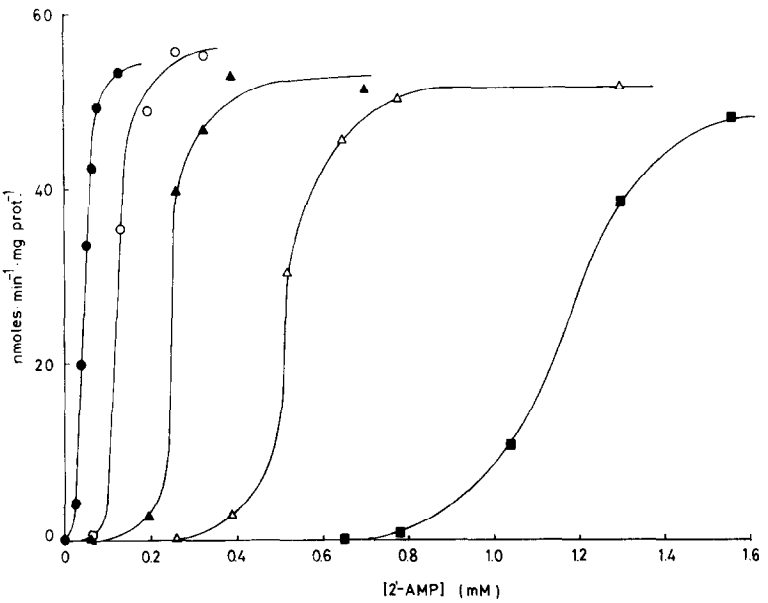

Fig. 2. Activation of the forward reaction by 2´-AMP in the presence of varying concentrations of Ca^{2+}. The concentrations of Ca^{2+} were: ■, no Ca^{2+}; Δ, 20 μM; ▲, 60 μM; o, 100 μM; ●, 200 μM. Other conditions were as described in Fig. 1.

AMP which is decreasing with increasing concentration of Ca^{2+} and vice versa. Similarly, the activating effect of NADPH on the forward reaction was strongly Ca^{2+} dependent (Fig. 3). It may be seen that at low concentrations of Ca^{2+} the enzyme is

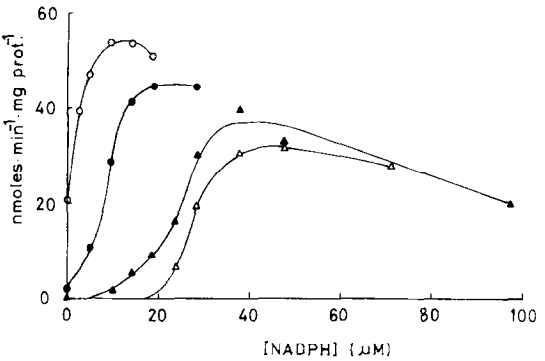

Fig. 3. Activation of the forward reaction by NADPH in the presence of varying concentrations of Ca^{2+}. The concentrations of Ca^{2+} were: Δ, 80 μM; ▲, 100 μM; ●, 200 μM; o, 300 μM. Other conditions were as described in Fig. 1.

inhibited. This inhibition cannot be overcome simply by raising the concentration of $NADP^+$, since under these conditions the latter substrate is strongly inhibitory (1,6,8, cf. also Discussion).

Both in the absence and in the presence of NADPH or 2′-AMP, Ca^{2+} could be replaced wholly or partially by other di- and monovalent cations, the order of efficiency being Mn^{2+} > Mg^{2+} > K^+. Compared to Ca^{2+}, about ten times higher concentrations of Mn^{2+} were required to obtain the same degree of activation. Saturation with Mg^{2+} or K^+ did not result in full activation. Moreover, these ions inhibited the Ca^{2+}-induced activation, probably by competing for the same binding site.

Fig. 4 A shows the effect of varying concentrations of NADPH on the reverse reaction, in the absence and in the presence of saturating concentrations of Ca^{2+}. In the absence of Ca^{2+} the enzyme was activated by increasing concentrations of NADPH. The addition of Ca^{2+} restored the Michaelis-Menten dependence, but did not alter significantly the maximal rate. The effect of Ca^{2+} on the forward reaction assayed at varying concentrations

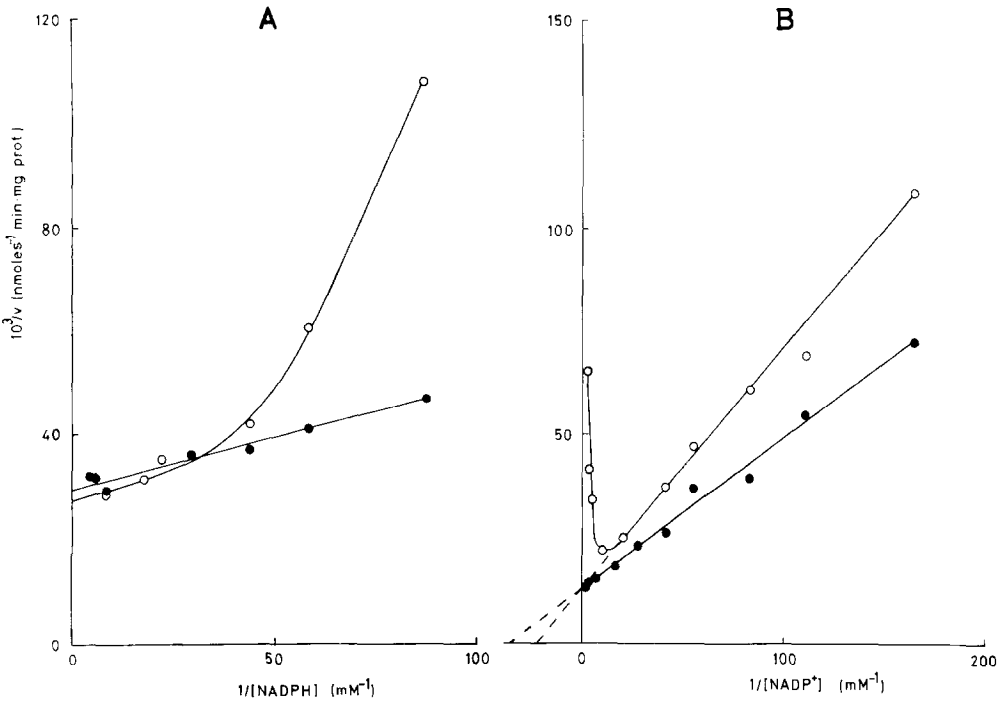

Fig. 4. Effect of Ca^{2+} on the reverse (A) and forward (B) reaction as a function of the NADP(H) concentration. The concentrations of Ca^{2+} were: (A), o, no Ca^{2+}; ●, 2 mM; (B), o, 300 µM; ●, 2 mM. The concentrations of the substrates were: (A) 110 µM NAD^+; (B) 100 µM NADH.

of $NADP^+$ is shown in Fig. 4 B. In the absence of Ca^{2+} the enzyme is strongly inhibited (cf. refs. 1, 6, 8). Increasing levels of Ca^{2+} led to an increase in the affinity for $NADP^+$, although high concentrations of $NADP^+$ were still inhibitory. Saturation with Ca^{2+} completely abolished the inhibitory effects of $NADP^+$.

Recently, Kaplan and co-workers (8) investigated the activating effect of a variety of compounds structurally analogues to NADP. They found that the 2´-phosphate group in the adeno-

Table 1

Activation of the forward reaction by different adenosine derivatives

Adenosine derivative (300 μM)	% activity[x/]
2'-AMP	100
-	5
3'-AMP	13
3'-5'-cyclic AMP	14
5'-AMP	10
Adenosine	5
2'-3'-cyclic AMP	94
CoA	64
acetyl-CoA	6

Reaction conditions were as described in Fig. 1.

[x/] measured in the presence of 200 μM Ca^{2+}.

sine moiety was essential for activation. These results are partly confirmed by the data shown in Table 1, obtained in the presence of non-saturating concentrations of Ca^{2+}. In addition, certain 3'-phospho-adenosine derivatives, e.g., CoA, were found to be activators. Since the latter finding may be of physiological significance, an investigation of the effects of various CoA derivatives is in progress.

DISCUSSION

The present data show that nicotinamide nucleotide transhydrogenase from Pseudomonas aeruginosa is strongly influenced by Ca^{2+}. Other di- and monovalent cations also act as activators, although the concentrations required for half-maximal stimulation are at least ten times that for Ca^{2+}. In addition, the other activating ions are antagonistic to Ca^{2+}. Previously, van den Broek et al. (9,11,15) have reported slight effects of Mg^{2+} and Ca^{2+} on nicotinamide nucleotide transhydrogenase from Azoto-

bacter vinelandii. The nature of these effects were, however, not further investigated.

Seemingly, the effect of Ca^{2+} differs depending on which direction of transhydrogenation is studied. In the absence of Ca^{2+} the reverse reaction exhibits a co-operative, sigmoidal, NADPH dependence, which is converted into a Michaelis-Menten hyperbola upon addition of saturating concentrations of Ca^{2+}. Other nucleotides structurally resembling NADPH, e.g., 2´-AMP, also activate, presumably by occupying the same site as NADP(H) (cf. ref. 6). Compounds lacking the 2´(or 3´)-phosphate group, such as NAD(H) and related compounds, are inactive. In contrast, in the absence of Ca^{2+}, $NADP^+$ does not stimulate but inhibits the reverse reaction (cf. ref. 6). With increasing concentrations of Ca^{2+} this inhibition is abolished, suggesting that $NADP^+$ prevents the co-operative effect of NADPH. This phenomenon is further illustrated by the effect of varying concentrations of $NADP^+$ on the forward reaction. As shown previously (6), $NADP^+$ inhibits severely the NADPH (or 2´-AMP)-activated forward reaction. When NADPH is replaced by saturating concentrations of Ca^{2+}, the $NADP^+$ dependence becomes hyperbolic. In the presence of non-saturating concentrations of Ca^{2+} the affinity for $NADP^+$ is decreased and, in addition, higher concentrations of $NADP^+$ are inhibitory. Thus, at low concentrations, $NADP^+$ appears to be allosterically inactive, but competes with NADPH for binding to the enzyme. This picture is, indeed, consistent with the classical allosteric model of the K-system type, originally proposed by Monod et al. (18), where the binding of NADPH induces a positive, co-operative, homotropic effect. According to this model, Ca^{2+} is a positive effector which, when bound to a specific allosteric site, has a heterotropic effect. Alternatively,

Ca^{2+} may increase the affinity for NADP(H) directly by combining with the substrate. The possible existence of an NADPH-specific regulatory site (different from the substrate site) as well as a possible negative allosteric effect of $NADP^+$ at a high $[NADP^+]/[Ca^{2+}]$ ratio remain as yet an open question.

Physiologically, the Pseudomonas transhydrogenase (cf. ref. 6) may function in the transfer of hydrogens between the pools of NAD(H) and NADP(H). Since at low concentrations of Ca^{2+} NADPH and $NADP^+$ have opposite effects with respect to the activation of the enzyme, it appears that the prevailing redox level of NADP and the intracellular concentration of free Ca^{2+} would efficiently regulate the activity of the transhydrogenase.

The present investigation has been carried out with a particle preparation of the P. aerug. transhydrogenase. A thorough investigation of the allosteric properties and reaction mechanism of the crystallized transhydrogenase, prepared according to Cohen and Kaplan (6), is in progress. Preliminary results indicate that the properties of the latter enzyme do not differ significantly from the particulate enzyme.

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