NAD-specific isocitrate dehydrogenase from bovine heart

Interaction with Ca^{2+} chelators

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The activity of NAD-specific isocitrate dehydrogenase was inhibited by EDTA, EGTA and other nitrogen-containing polycarboxylate Ca^{2+} chelators in the absence and in the presence of ADP by a mechanism that could not be attributed solely to the removal of free Ca^{2+} . Carboxymethyltartronate (2-oxapropane-1,1,3-tricarboxylate), an oxygen ether polycarboxylate chelator, did not inhibit when ADP was absent. The activation by ADP, a positive effector of the enzyme, decreased with increasing concentration of carboxymethyltartronate, paralleling the removal of free Ca^{2+} by this chelator. The following were found when free Ca^{2+} was decreased to negligible concentrations (5–50 nM) with carboxymethyltartronate. (1) Free Ca^{2+} enhanced, but was not absolutely required for, activation by ADP. (2) Activation of enzyme activity by magnesium citrate neither required nor was increased by Ca^{2+} when ADP was absent. However, the potentiation of citrate activation by ADP was facilitated by free Ca^{2+} . (3) The reversal of NADPH inhibition of enzyme activity by ADP did not absolutely require Ca^{2+} , but it was enhanced by free Ca^{2+} . (4) The inhibition of enzyme activity by NADH was not reversed by ADP either with or without Ca^{2+} .

NAD-specific isocitrate dehydrogenase (EC 1.1.1.41) from mammalian tissues is activated by free Ca^{2+} in the presence of the allosteric effector ADP, but not without ADP (Denton *et al.*, 1978; Aogaichi *et al.*, 1980). However, EGTA, which was used to regulate the concentration of Ca^{2+} in such studies (Denton *et al.*, 1978), inhibited enzyme activity with and without ADP (Aogaichi *et al.*, 1980). To minimize the concentration of endogenous Ca^{2+} without the use of EGTA in studies with homogeneous NAD-specific isocitrate dehydrogenase, careful attention had to be paid to the quality of the reagents, and high concentrations of isocitrate were used to regulate the concentrations of free Ca^{2+} at the values desired. Under such conditions an $[\text{S}]_{0.5}$ value of 0.3–0.6 μM for Ca^{2+} activation could be determined (Aogaichi *et al.*, 1980).

Under the experimental conditions above (Denton *et al.*, 1978; Aogaichi *et al.*, 1980), the Ca^{2+} -facilitated activation by ADP was attributable to a lowering of the $[\text{S}]_{0.5}$ of the substrate magnesium isocitrate. However, ADP has been found more recently also to modify the activation of the

enzyme by magnesium citrate (Gabriel & Plaut, 1984a) and the reversal of inhibition by NADPH (Gabriel & Plaut, 1984b). It could be demonstrated that Ca^{2+} facilitated the ADP-mediated reversal of inhibition by NADPH (Gabriel & Plaut, 1984b), but it was uncertain whether Ca^{2+} was absolutely required for this effect. A Ca^{2+} chelator that neither inhibits nor activates the enzyme was required to show unequivocally whether or not the activation by citrate or reversal of NADPH inhibition was mediated by Ca^{2+} . The effect on the activity of the enzyme of a number of ligands that chelate Ca^{2+} preferentially over Mg^{2+} was therefore examined in the present study. One of the chelators, CMT, had the properties desired and could be used to examine the activity of the enzyme at negligible concentrations of free Ca^{2+} .

Experimental

NAD-specific isocitrate dehydrogenase from bovine heart was purified and stored as described previously (Giorgio *et al.*, 1970; Fan *et al.*, 1975). Initial velocities are expressed as units/mg of protein, where 1 unit of enzyme activity is defined as the amount of enzyme required to catalyse the production of 1 μmol of NADH/min at 25°C. All

Abbreviation used: CMT, carboxymethyltartronic acid (2-oxapropane-1,1,3-tricarboxylate).

reaction mixtures contained 0.167M-Hepes/NaOH at pH 7.4.

CMT was a gift from Dr. Thomas Tolbert from Monsanto Co. (St. Louis, MO, U.S.A.). EGTA, 2-hydroxyethylamine-*NN*-diacetic acid and nitrilotriacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), EDTA and iminodiacetic acid were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and $\alpha\alpha'$ -oxydiacetic acid was from Chem Service (West Chester, PA, U.S.A.).

Although the enzyme is stereospecific for *D*-*threo*-isocitrate, the racemic mixture *DL*-*threo*-isocitrate was used in all the experiments presented, since this material was more readily available. If the natural enantiomer were used, the corresponding apparent $[S]_{0.5}$ value would be equivalent to one-half that for magnesium *DL*-isocitrate.

The free and metal-ion-chelated concentrations of isocitrate, citrate and ADP were maintained at the concentrations indicated in the text and Figures by adjusting the total ligand, Mg^{2+} and Ca^{2+} concentrations. The ligand and metal ion concentrations were calculated as described previously (Gabriel & Plaut, 1984b) by using the additional magnesium and calcium stability constants for the chelators given in Table 1. In this manner one can account for the metal-ion binding to the chelators.

Total endogenous calcium was determined by atomic absorption (Perkin Elmer model 4000 instrument). The free Ca^{2+} concentration in the presence of chelators was calculated as described above.

Kinetic constants were calculated as follows. Since plots of percentage inhibition versus ligand concentration for the chelators listed in Table 1 and NAD(P)H were hyperbolic, the inhibition constant $K_{i(app.)}$ was calculated (at the concentration of substrate stated) by fitting data for percentage inhibition (%I) versus inhibitor concentration ([I]) to eqn. (1):

$$\%I = \frac{(\text{maximal percentage inhibition} \times [I])}{(K_{i(app.)} + [I])} \quad (1)$$

The numbers of data points used in the calculation of $K_{i(app.)}$ and other kinetic constants are given in parentheses after the value of the constant in the text.

The apparent $[S]_{0.5}$ for magnesium *DL*-isocitrate was determined by fitting data for initial velocity (v) versus substrate concentration ($[S]$) to the Hill equation (eqn. 2):

$$v = \frac{V[S]^h}{(K + [S]^h)} \quad (2)$$

with the aid of the computer programs of Cleland (1963), where h is the Hill coefficient ($h = 1.5$ in the present study) and K is defined as $([S]_{0.5(app.)})^h$.

Kinetic constants for magnesium citrate (MgC) activation were determined by fitting data for initial velocity versus magnesium citrate concentration to eqn. (3):

$$v - v_0 = \frac{V[MgC]^h}{K + [MgC]^h} \quad (3)$$

where v_0 is activity in the absence of citrate and h and K are defined above. When ADP and Ca^{2+} were present, the kinetic behavior was hyperbolic ($h = 1$), whereas sigmoidal plots of $(v - v_0)$ versus $[MgC]$ were obtained with ADP alone ($h = 1.2$) and ADP absent ($h = 1.4$).

Where applicable, when the presence and absence of a calcium chelator had no apparent effect on the value of a kinetic constant, the average value of the constant was calculated by fitting all data points, with and without the chelator present, to the appropriate kinetic equation.

Results and discussion

Enzyme-chelator interactions

The effect on enzyme activity of various concentrations of EGTA and other chelators was examined at subsaturating substrate concentrations with and without ADP, as specified in particular experiments. Except where noted, Ca^{2+} was not added and the values of endogenous Ca^{2+} are reported for each of the experiments. The effects of the chelators are summarized in Table 1 as (i) the concentration of ligand required for half-maximal inhibition ($K_{i(app.)}$) and (ii) the percentage inhibition where further addition of ligand did not produce an additional effect (maximum inhibition).

The inhibition by increasing concentrations of EGTA reached a plateau, which was maximal at about 100 μ M-EGTA (Fig. 1a) either with ADP (curve 1) or without ADP (curve 2); no further decrease in activity was obtained by increasing EGTA up to 5 mM (results not shown). Since Ca^{2+} enhanced the activation of the enzyme by ADP (Denton *et al.*, 1978; Aogaichi *et al.*, 1980), inhibition as a result of the removal of free Ca^{2+} by EGTA would be expected when ADP was present (Fig. 1a, curve 1). However, no stimulation of activity by added Ca^{2+} could be demonstrated in the absence of ADP (Denton *et al.*, 1978; Aogaichi *et al.*, 1980), and the present finding of inhibition by EGTA without ADP (Fig. 1a, curve 2) was consistent with the previous proposal that EGTA can interact with the enzyme by a mechanism in

Table 1. Inhibition of NAD-specific isocitrate dehydrogenase activity by Ca²⁺ chelators

Reaction mixtures contained 0.2mM total DL-isocitrate, 0.5mM-NAD⁺, 4.8 μM endogenous total Ca²⁺ and 0.167M-Hepes/NaOH at pH 7.4; the concentration of magnesium DL-isocitrate was maintained constant at 0.066mM by the addition of MgSO₄. When ADP was present (+), 0.5mM total ADP corresponding to 0.2mM free ADP³⁻ was used. The stability constants at pH 7.4 for the magnesium chelate [K(Mg)] and the calcium chelate [K(Ca)] were calculated from literature values (Portzehl *et al.*, 1964; Sillen & Martell, 1971; Martell & Smith, 1974*a,b*). Kinetic constants were determined as described in the Experimental section by using triplicate determinations at six ligand concentrations.

Ligand	K(Mg) (mM ⁻¹)	K(Ca) (mM ⁻¹)	Percentage of maximum inhibition	K _{i(app)} (μM)	Presence of ADP
EGTA	0.08	2.98 × 10 ⁴	82.3 ± 1.2	2.1 ± 0.3	+
EGTA	0.08	2.98 × 10 ⁴	61.5 ± 2.5	6.8 ± 1.3	-
EDTA	684.1	5.43 × 10 ⁴	82.4 ± 2.6	10.6 ± 1.1	+
EDTA	684.1	5.43 × 10 ⁴	69.2 ± 1.9	2.1 ± 0.5	-
Nitrilotriacetate	1.07	9.95	55.9 ± 1.3	2.6 ± 0.4	+
2-Hydroxyethylamine- NN-diacetate	0.13	1.95	43.4 ± 1.5	1.6 ± 0.5	+
Iminodiacetate	0.01	0.004	20.1 ± 1.2	27.7 ± 3.3	+
CMT	0.589	41.7	55.9 ± 5.8	50.9 ± 15.2	+
CMT	0.589	41.7	None	-	-

Fig. 1. Effect of Ca²⁺ chelators on NAD-specific isocitrate dehydrogenase activity with and without ADP

Reaction mixtures contained 0.2mM total DL-isocitrate and 0.5mM-NAD⁺; the concentration of magnesium DL-isocitrate was maintained constant at 0.066mM by addition of increasing concentrations of MgSO₄. The data are presented as means ± corresponding s.d. for two or three determinations. Activity with 0.2mM free ADP³⁻ (curve 1) and without ADP (curve 2) is shown. The calculated free [Ca²⁺] (see the Experimental section) at each chelator concentration is given in the upper scale. (a) Inhibition by EGTA; total endogenous Ca²⁺ was 4.8 μM. (b) Effect of CMT on enzyme activity; total endogenous Ca²⁺ was 1.4 μM.

addition to Ca²⁺ chelation (Aogaichi *et al.*, 1980). This suggestion is supported by the following additional observations. (i) The stability constants for Ca²⁺ chelation and the kinetic parameters for enzyme inhibition were similar for EGTA and EDTA (Table 1). As with EGTA (Fig. 1*a*), the inhibition by EDTA in the presence of ADP could only be partially prevented when the free Ca²⁺ concentration with various EDTA concentrations was maintained at the same value as in the absence of EDTA (Fig. 2*a*, curve 2). (ii) A number of other nitrogen-containing polycarboxylic acids, related

in chemical structure to EGTA, were also found to be inhibitors of the enzyme. If chelation of Ca²⁺ were the only effect of these compounds on enzyme activity, one would have expected the maximal extent of inhibition to be the same for all of the chelators. However, when compared in the presence of ADP, the values for maximum inhibition were found to vary substantially between compounds (Table 1). Furthermore, even though the stability constants for Ca²⁺ chelation differed by several orders of magnitude among the compounds, the values of K_{i(app)} were relatively

similar. This discrepancy became particularly apparent when one estimated from the relevant stability constants the concentrations of chelators required to lower the concentration of free Ca^{2+} to $0.6\text{ }\mu\text{M}$, the value that should give half-maximal enhancement of the activation by ADP (i.e. an $[\text{S}]_{0.5}$ value for Ca^{2+} of $0.6\text{ }\mu\text{M}$). For example, the calculated concentrations of 2-hydroxyethylamine-*NN*-diacetic acid (4 mM) and iminodiacetate (1.5 M) required to produce this effect under the conditions shown in Table 1 were larger by many orders of magnitude than the corresponding observed values of $K_{i(\text{app.})}$ for these chelators. These results indicated that, even in the presence of ADP, there was a direct interaction between the enzyme and micromolar concentrations of nitrogen-containing polycarboxylate derivatives, resulting in negative modification of activity by a mechanism or mechanisms in addition to removal of free Ca^{2+} by chelation.

In a survey of Ca^{2+} chelators differing in structure from EGTA and EDTA it was observed that $\alpha\alpha'$ -oxydiacetate did not inhibit the enzyme in the absence of ADP. However, since $\alpha\alpha'$ -oxydiacetate binds Ca^{2+} rather weakly, the structurally related oxygen ether carboxylate CMT (Hallcher *et al.*, 1979), which is an effective Ca^{2+} chelator (Martell & Smith, 1974b), was tested. CMT decreased enzyme activity in the presence of ADP, but not without ADP (Fig. 1b); the inhibition was fully reversible by the addition of Ca^{2+} (Fig. 2b). In addition, it could be calculated that at an initial concentration of $1.4\text{ }\mu\text{M}$ - Ca^{2+} it should require $44\text{ }\mu\text{M}$ -CMT to decrease free Ca^{2+} to a concentration resulting in half-maximal activation in the

presence of ADP (Fig. 1b). This was in reasonable agreement with the experimentally determined value of $K_{i(\text{app.})}$ for CMT (Table 1).

It appeared from earlier experiments, where the concentration of free Ca^{2+} was regulated by adjusting the concentration of free isocitrate, that free Ca^{2+} was not absolutely required for enzyme activation by ADP (Aogaichi *et al.*, 1980). This is in agreement with the present finding that a low degree of ADP activation persisted even when the concentration of free Ca^{2+} was decreased to $5\text{--}50\text{ nM}$ by $1.0\text{--}10\text{ mM}$ -CMT (cf. curves 1 and 2 in Fig. 1b). It would be expected that the activation by ADP, even in the virtual absence of free Ca^{2+} , should still be due to its effect on the K_m of magnesium isocitrate. This was found to be the case. Thus, in an experiment where the magnesium isocitrate concentration was varied, endogenous Ca^{2+} was $1.6\text{ }\mu\text{M}$ and the concentrations of NAD^+ (0.25 mM) and free isocitrate (10.0 mM) were constant, the apparent $[\text{S}]_{0.5}$ for magnesium DL-isocitrate was $0.95 \pm 0.06\text{ mM}$ (35) without ADP, $0.36 \pm 0.02\text{ mM}$ (16) with 0.2 mM -ADP and $0.52 \pm 0.02\text{ mM}$ (13) with 0.2 mM -ADP when the concentration of free Ca^{2+} was decreased to negligible values with 10 mM -CMT (results not shown).

Effect of free Ca^{2+} on activation by magnesium citrate

NAD-specific isocitrate dehydrogenase from heart was activated by lower concentrations of citrate with ADP than without ADP. Furthermore, it was shown that there is an interdependence of the interaction of these activators with the enzyme and that either activator increased the

Fig. 2. Effect of Ca^{2+} on inhibition of NAD-specific isocitrate dehydrogenase activity by Ca^{2+} chelators. Reaction mixtures contained 0.066 mM -magnesium DL-isocitrate, 0.2 mM total DL-isocitrate, 0.5 mM - NAD^+ and 0.2 mM free ADP^{3-} . The data are presented as means $\pm$ corresponding s.d. for three determinations. (a) EDTA: Curve 1, total endogenous Ca^{2+} was $4.8\text{ }\mu\text{M}$; free $[\text{Ca}^{2+}]$ at each chelator concentration is given in upper scale; curve 2, the free Ca^{2+} concentration was maintained at $4.2\text{ }\mu\text{M}$ by the addition of CaCl_2 . (b) CMT: Curve 1, initial free Ca^{2+} was $20.0\text{ }\mu\text{M}$; free Ca^{2+} concentration at each chelator concentration is given in upper scale; Curve 2, the free Ca^{2+} concentration was maintained at $20.0\text{ }\mu\text{M}$ by the addition of CaCl_2 .

affinity of the enzyme for the substrate magnesium isocitrate (Gabriel & Plaut, 1984a). It was not possible to demonstrate unequivocally in the earlier experiments whether the synergistic relationship between magnesium citrate and ADP on enzyme activation was modified further by Ca^{2+} . It could be shown in the present study that activation of the enzyme by magnesium citrate without ADP was not affected by 1.0mM-CMT when initial velocities were measured at subsaturating concentrations (Table 2). Under these experimental conditions, about 98% of the total endogenous Ca^{2+} (1.4–2.7 μM) should have been chelated by 1.0mM-CMT. Hence free Ca^{2+} appears not to enhance or to be required for the activation by magnesium citrate when ADP is absent. The $[\text{S}]_{0.5}$ for magnesium citrate activation was about 100-fold smaller with ADP than without ADP at micromolar concentrations of endogenous Ca^{2+} (CMT absent) (Table 2), which is consistent with previous results (Gabriel & Plaut, 1984a). The effect of ADP on citrate activation was diminished when free Ca^{2+} was removed with CMT; nevertheless the $[\text{S}]_{0.5}$ for magnesium citrate at these negligible free Ca^{2+} concentrations was still about 10-fold smaller with ADP than without ADP (Table 2). These results suggest that free Ca^{2+} enhances, but is not absolutely required for, the ADP facilitation of the activation of the enzyme by magnesium citrate.

Effect of Ca^{2+} on NAD(P)H inhibition

The inhibition by NADPH of the enzyme was reversed competitively by magnesium isocitrate with ADP, but not without ADP (Gabriel & Plaut, 1984b). Furthermore, it was shown in the earlier experiments that at constant concentrations of subsaturating substrates, and NADPH and ADP, addition of Ca^{2+} resulted in a marked increase in the inhibition constant for NADPH. An apparent

$[\text{S}]_{0.5}$ for Ca^{2+} of $48 \pm 11 \mu\text{M}$ for modulation of the reversal of NADPH inhibition by ADP was observed under these conditions. In the present experiments it could be demonstrated that, although Ca^{2+} facilitated the reversal by ADP of NADPH inhibition, free Ca^{2+} was not absolutely required for this action of ADP, since the decrease in the concentration of free Ca^{2+} to negligible values with 1.0mM-CMT (the endogenous total Ca^{2+} concentration in the assay was approx. 2 μM) did not result in a change in $K_{i(\text{app.})}$ for NADPH inhibition in the presence of 0.2mM free ADP^{3-} [the average $K_{i(\text{app.})}$ was $30.7 \pm 2.7 \mu\text{M}$ (30)].

It was demonstrated previously that, at constant subsaturating concentrations of magnesium isocitrate and NAD^+ , the $K_{i(\text{app.})}$ value for NADH inhibition was not affected by added Ca^{2+} either with or without ADP. However, it seemed possible that the low endogenous Ca^{2+} concentration in the assay (approx. 2 μM) may have been large enough to modify the inhibition by NADH (Gabriel & Plaut, 1984b). When these experiments were repeated under identical conditions, except that the inhibition constants for NADH were compared in the presence and in the absence of 1 mM-CMT, the average values of $K_{i(\text{app.})}$ for NADH at endogenous and low free Ca^{2+} concentrations were $5.5 \pm 0.4 \mu\text{M}$ (20) with 0.2mM- ADP^{3-} and $5.1 \pm 0.6 \mu\text{M}$ (18) without ADP. Thus, in contrast with the situation with NADPH, the kinetic constants for inhibition by NADH, as measured in the present and previous experiments, were unaffected by ADP or by Ca^{2+} .

Conclusions

Micromolar concentrations of the commonly used calcium buffer EGTA and other nitrogen-containing polycarboxylate calcium chelators inhibited NAD-specific isocitrate dehydrogenase with or without ADP in a manner that could not be

Table 2. Effect of free Ca^{2+} on magnesium citrate activation of NAD-specific isocitrate dehydrogenase. Reaction mixtures contained 0.18mM-magnesium DL-isocitrate, 0.45mM free DL-isocitrate, 0.25mM- NAD^+ and 0.167M-Hepes/NaOH at pH 7.4. When present, the concentrations of CMT and free ADP^{3-} were 1.0mM and 0.2mM respectively. The total endogenous Ca^{2+} concentrations were in the range 1.4–2.7 μM . When CMT was present, the endogenous free Ca^{2+} concentration should be less than 0.06 μM . Kinetic constants were calculated as described in the Experimental section. Velocities in the absence of citrate and at a saturating concentration of citrate are designated as v_0 and V respectively.

Kinetic constants	No ADP	ADP present, CMT absent	ADP and CMT present
v_0 (units/mg)	$0.32 \pm 0.02^*$	3.14 ± 0.1	0.73 ± 0.03
V (units/mg)	$3.8 \pm 0.2^*$	14.1 ± 0.4	10.9 ± 0.4
$[\text{S}]_{0.5}$ (magnesium citrate) (mM)	$3.8 \pm 0.5^*$	0.033 ± 0.003	0.43 ± 0.03
Data points used in calculation	31	10	27

* The results were the same with or without CMT.

correlated solely with the removal of free Ca^{2+} (Table 1). The enzyme was not inhibited by the oxygen ether calcium chelator CMT in the absence of ADP (Fig. 1, curve 2) and, in contrast with EGTA, with ADP its activity was fully retained with increasing concentrations of CMT when the concentration of free Ca^{2+} was kept constant at the initial values (compare curve 2 in Figs. 2a and 2b).

By the use of CMT to regulate the concentration of free Ca^{2+} it was possible to demonstrate the following unequivocally. (i) Free Ca^{2+} stimulated, but was not absolutely essential for, activation by ADP. This is apparent from a comparison of curves 1 and 2 in Fig. 1(b) when free Ca^{2+} was decreased to between 0.005 and 0.05 μM with CMT. This was supported by the further observation that the value of the apparent $[\text{S}]_{0.5}$ of magnesium isocitrate was about twice as large without ADP as with 0.2 mM-ADP when free Ca^{2+} was decreased to negligible values with CMT. These results are consistent with previous experiments where the concentration of free Ca^{2+} was regulated by changing concentrations of free isocitrate (Aogaichi *et al.*, 1980). (ii) The enhancement by ADP of magnesium citrate activation (Gabriel & Plaut, 1984a) was facilitated by Ca^{2+} , but free Ca^{2+} was not absolutely required for this effect (Table 2). Thus even at negligible free Ca^{2+} concentration ($<0.06 \mu\text{M}$) the apparent $[\text{S}]_{0.5}$ for magnesium citrate activation was still about 9-fold smaller with ADP than without ADP (Table 2). (iii) Reversal of NADPH inhibition by ADP had been shown previously to be enhanced by Ca^{2+} with an apparent K_m for Ca^{2+} of about 48 μM (Gabriel & Plaut, 1984b). The present finding that the value of apparent K_i for NADPH in the

presence of ADP was not changed substantially when the concentration of endogenous free Ca^{2+} was decreased from about 1.1 to 0.06 μM with CMT is consistent with the previous data and with the view that Ca^{2+} positively modifies, but is not absolutely required for, the reversal of NADPH inhibition by ADP.

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