Inhibition of Bovine Heart NAD-Specific Isocitrate Dehydrogenase by Reduced Pyridine Nucleotides: Modulation of Inhibition by ADP, NAD^+, Ca^{2+}, Citrate, and Isocitrate†

Jerome L. Gabriel and Gerhard W. E. Plaut*

ABSTRACT: The activity of NAD-dependent isocitrate dehydrogenase from bovine heart was inhibited by NADH (apparent K_i about 4.3 μM) and NADPH (K_i about 9.8 μM) at subsaturating substrate concentrations at pH 7.4. The inhibition by NADH or NADPH was reversed competitively by magnesium isocitrate in the presence of ADP, but not without ADP. Reversal of inhibition by NADH or NADPH with respect to NAD^+ was competitive or of the linear mixed type depending on whether ADP was absent or present. ADP^{3-} (0.2 mM) increased the K_i(app) for NADPH from 9.8 to 27.1 μM; further addition of Ca^{2+} (0.2 mM) raised the K_i(app) to 127 μM. For the modification of NADPH inhibition by ADP, $S_{0.5}$ for Ca^{2+} was approximately 48 μM. This compares to the K_m for Ca^{2+} of 0.3–1 μM for the activation of the enzyme without NADPH [Denton, R. M., Richards, D. A., & Chin, J. G. (1978) *Biochem. J. 176*, 899–906; Aogaichi, T., Evans, J., Gabriel, J., & Plaut, G. W. E. (1980) *Arch. Biochem. Biophys. 204*, 350–360]. ADP did not affect the K_i for NADH. Magnesium citrate, which was about 100-fold more effective as a positive modifier of the enzyme with ADP than without ADP [Gabriel, J. L., & Plaut, G. W. E. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2082], reversed competitively the inhibition by NADPH in the presence of ADP, but not without ADP. Magnesium citrate did not reverse NADH inhibition. The results suggest that the reversal of NADPH inhibition by NAD^+ and by the ADP-dependent action of magnesium isocitrate and magnesium citrate could be significant factors in the regulation of the activity of the enzyme in mitochondria.

Inhibition by NADH or NADPH of NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41) has been reported for the soluble enzyme from heart (Chen & Plaut, 1963), liver (Plaut & Aogaichi, 1968), and Ehrlich ascites tumor (Stein et al., 1967). With the bovine heart enzyme, the inhibition constant for NADH obtained by kinetic measurements (Chen & Plaut, 1963) was appreciably different from the binding constant determined by fluorescence (Harvey et al., 1972). However, the conditions of incubation were different in the kinetic and binding experiments. Binding of NADH to enzyme was examined in the absence of isocitrate, divalent metal ion activator, or ADP (Harvey et al., 1972) whereas the kinetics of inhibition were investigated with nearly saturating substrate concentrations (5.3 mM total DL-isocitrate, 1.33 mM Mn^{2+}, 0.33 mM NAD^+, and 0.67 mM ADP at pH 7.2). Under the latter conditions, inhibition by NADH was competitive with NAD^+; NADPH appeared not to inhibit the initial rate of the reaction of the heart enzyme but seemed to potentiate inhibition by NADH (Chen & Plaut, 1963).

NAD-isocitrate dehydrogenase appears to have an important role in regulating flux through the citric cycle in mammalian tissues. Regulation of flux through this enzyme by the NADH/NAD^+ ratio may be quantitatively more important than positive modulation by ADP, as determined in studies with the soluble enzyme from porcine and rat liver (Plaut & Aogaichi, 1968; Lenartowicz et al., 1976), with intact mitochondria from rat liver (Williamson et al., 1969; König et al., 1969), rat heart (LaNoue et al., 1970), and rabbit heart (Hansford & Johnson, 1975), and with isolated perfused rat heart (Hiltunen & Hassinen, 1977). The reasons underlying these conclusions have been reviewed (Williamson & Cooper, 1980; Hansford, 1980). Conditions which decreased the NADPH/$NADP^+$ ratio in isolated coupled rat liver mitochondria increased the rate of isocitrate oxidation (Plaut & Smith, 1977; Smith & Plaut, 1979). For example, the enhancement of isocitrate oxidation by the addition of NH_4Cl persisted even when NADP-isocitrate dehydrogenase was inhibited by α-methylisocitrate. Since NH_4Cl did not appear to decrease the already low NADH levels, it was suggested that under these conditions NADPH rather than NADH may be a significant factor in regulating isocitrate oxidation at the level of NAD-isocitrate dehydrogenase (Smith & Plaut, 1979). The present observations with the soluble enzyme further support such a role of NADPH.

Since the previous studies were done, a number of additional factors have been found to affect the activity of NAD-isocitrate dehydrogenase. It has been found that Ca^{2+} potentiates activation of the enzyme by ADP (Denton et al., 1978; Aogaichi et al., 1980) and that Ca^{2+} or ADP enhances activation of the enzyme by citrate (Plaut, 1981; Gabriel & Plaut, 1983). The present studies were done to gain a better understanding of the effects of these activators and of substrate concentration on inhibition by the reduced pyridine nucleotides at the level of the purified enzyme.

Experimental Procedures

Materials. DL-*threo*-Isocitric acid lactone from Aldrich was recrystallized from ethyl butyrate and hydrolyzed as previously described (Gabriel & Plaut, 1980). NAD^+, NADH, and ADP were obtained from Sigma, NADPH was from Boehringer-Mannheim, and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes),[1] recrystallized as described previously (Gabriel & Plaut, 1980), was from Research Organics.

† From the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. *Received October 12, 1983.* This work was supported in part by Grant AM 15404 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MI, magnesium DL-isocitrate; Ic, DL-isocitrate; MC, magnesium citrate; CaC, calcium citrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Table I: Kinetic Constants for NAD(P)H Inhibition[a]

coenzyme	$[ADP^{3-}]_{free}$ (mM)	$[Ca^{2+}]_{free}$ (mM)	coenzyme K_i(app) (μM)
NADH	0.0	0.0	6.3 ± 0.8
NADH	0.2	0.0	3.5 ± 0.1
NADH	0.0	0.2	2.9 ± 0.3
NADH	0.2	0.2	4.3 ± 0.2
NADPH	0.0	0.0	9.8 ± 1.1
NADPH	0.2	0.0	27.1 ± 6.9
NADPH	0.0	0.2	7.2 ± 0.9
NADPH	0.2	0.2	125.5 ± 41.3

[a] Assay conditions were as described in Figure 1. In the absence of added calcium, 2.0 μM total endogenous Ca^{2+} was present. The The apparent K_i values were determined by fitting percent inhibition vs. inhibitor concentration data to the following equation: percent inhibition = (maximal percent inhibition × [inhibitor])/[K_i(app) + [inhibitor]]. When extrapolated to infinite inhibition concentrations, the maximal inhibition was 100% in all cases.

NAD-dependent isocitrate dehydrogenase was purified from lyophilized mitochondria from bovine heart (Giorgio et al., 1970) and stored as described previously (Fan et al., 1975). Other chemicals used were reagent grade and were obtained commercially.

Kinetics. All enzyme assay mixtures contained 0.167 M NaHepes at pH 7.4; other components have been indicated in the text, tables, and figures. Velocities were expressed as units per milligram of protein, where 1 unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of NADH per min at 25 °C. Protein concentration was determined from the absorbance at 278 nm by using an extinction coefficient of 0.53 mL mg^{-1} cm^{-1} (Fan & Plaut, 1974).

Ligand Concentrations. The free and metal-chelated concentrations of isocitrate, citrate, and ADP were maintained at the levels indicated in the text and figures by adjusting the total ligand, magnesium and calcium concentrations. The required concentrations were calculated by using a computer program described by Feldman & co-workers (Feldman et al., 1972), and the stability constants at pH 7.4 for the respective magnesium and calcium complexes for isocitrate (0.525 mM^{-1}, 0.457 mM^{-1}), ADP (1.36 mM^{-1}, 0.365 mM^{-1}), and citrate (1.78 mM^{-1}, 2.40 mM^{-1}), were calculated from the literature values (Grzybowski et al., 1970; Sillen & Martell, 1971; Gabriel et al., 1983). Metal binding to NADH and NADPH was disregarded in the ligand concentration calculations for the kinetic studies because, at the concentrations used, these nucleotides did not significantly affect the distribution of the other ligand species.

Results

Inhibition by NAD(P)H. The effects of NADH and NADPH were studied at subsaturating substrate concentrations [0.2 mM magnesium DL-isocitrate, 0.45 mM free DL-isocitrate(3−),[2] and 0.25 mM NAD^+] at pH 7.4. The positive modifier ADP had relatively little effect on the apparent K_i for NADH, with an average value of 4.3 μM with and without ADP (Figure 1A and Table I). At the low substrate concentrations used, inhibition by NADPH of initial velocities was observed (Figure 1B). However, in contrast to inhibition by NADH, the apparent K_i for NADPH was increased 3-fold by ADP, i.e., from approximately 10 to 30 μM (Table I).

[2] Isocitrate(3−) denotes the totally ionized nonchelated form of the substrate while isocitrate denotes the total isocitrate concentration regardless of ionization state or degree of metal chelation. ADP^{3-} and ADP are defined in a similar manner.

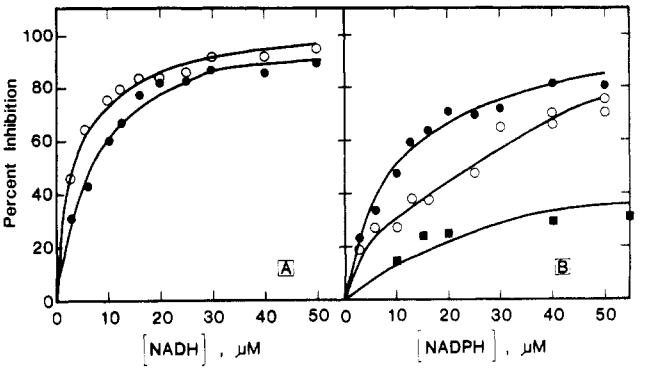

FIGURE 1: Effect of ADP on inhibition by NADH and NADPH. The results are presented as percent inhibition (I) as a function of NAD(P)H concentration. $I = 100(v_0 - v)/v_0$, where v_0 and v were the initial velocities in the absence and presence of inhibitor, respectively. Reaction mixtures contained 0.25 mM NAD^+, 0.2 mM magnesium DL-isocitrate, and 0.45 mM isocitrate(3−). (A) Inhibition by NADH in the absence (●) and presence (○) of 0.2 mM ADP^{3-}. (B) Inhibition by NADPH with the following additions: no ADP (●), 0.2 mM ADP^{3-} (○), and 0.2 mM ADP^{3-} with 0.2 mM free calcium (■).

Free Ca^{2+} activates the enzyme in the presence of ADP by lowering the apparent $S_{0.5}$ for magnesium isocitrate, but Ca^{2+} has no such effect without ADP (Denton et al., 1978; Aogaichi et al., 1980). When the effect of Ca^{2+} on inhibition by reduced pyridine nucleotides was examined, it was found that Ca^{2+} did not affect the inhibition by NADH with ADP; however, in the absence of ADP, the K_i for NADH decreased from 6.3 to 2.9 μM in the presence of calcium (Table I). The percent decrease in K_i was relatively large (54 ± 8%), but the magnitude of the change was quite small, 3.4 ± 0.9 μM NADH. Although inhibition by NADPH was unaffected by Ca^{2+} in the absence of ADP, the K_i for NADPH which was tripled by ADP without added Ca^{2+} was increased a further 5-fold with Ca^{2+} (Figure 1B and Table I).

The modulation of NADPH inhibition by ADP and its enhancement by Ca^{2+} suggested a possible competitive relationship between binding of reduced pyridine nucleotides and substrates to the enzyme. The effects of magnesium isocitrate and NAD^+ on the inhibition of NADH and NADPH were therefore examined. Inhibition by NADH in the presence of ADP was not affected by free calcium at concentrations ranging from 2.0 μM (endogenous calcium) to 0.25 mM. However, the possibility exists that the low endogenous Ca^{2+} levels present in the assay may have been large enough to produce a calcium-mediated effect on NADH inhibition. Removal of endogenous calcium by chelators such as EGTA has not been successful so far due to inhibition of the enzyme at micromolar chelator concentrations resulting from a direct chelator–enzyme interaction (Aogaichi et al., 1980).

Modulation of NAD(P)H Inhibition by Isocitrate and NAD^+. Without ADP, magnesium isocitrate did not affect inhibition by either NADH or NADPH (Figure 2A, curves 1 and 2). However, in the presence of ADP (Figure 2A, curves 3 and 4), the inhibition for either NADH or NADPH was competitive with respect to magnesium isocitrate (Figure 2B). At 0.039 mM, NADH and NADPH increased $S_{0.5}$ for magnesium DL-isocitrate from 0.76 mM to 2.0 and 1.4 mM, respectively. The Hill coefficient for magnesium isocitrate increased from 1.10 without NAD(P)H to 1.9 and to 1.6 with NADH and NADPH, respectively.

Free isocitrate had no effect on NADH inhibition either with ADP (Figure 3, curve 1) or without ADP (not shown). However, with magnesium isocitrate concentration constant at 0.2 mM, inhibition by NADPH was competitively reversed

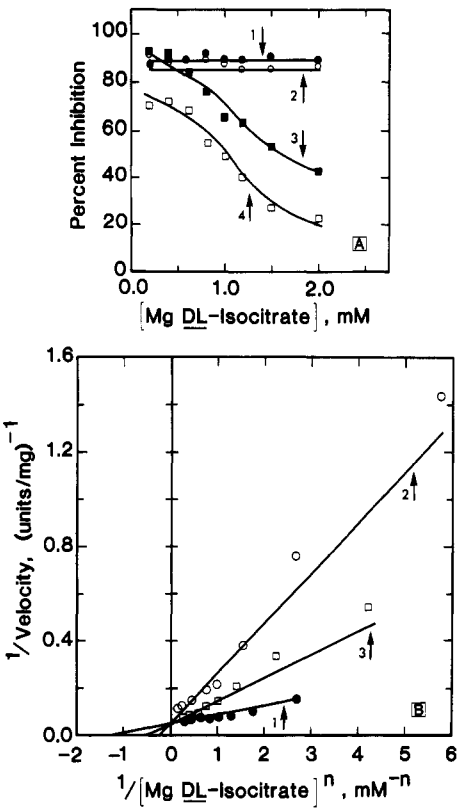

FIGURE 2: Effect of the concentration of the substrate magnesium isocitrate on NAD(P)H inhibition. The reaction mixtures contained 0.25 mM NAD^+ and 0.45 mM isocitrate(3–); magnesium isocitrate concentration varied. (A) Plot of percent inhibition vs. substrate concentration. Curve 1: Inhibition by 0.039 mM NADH without ADP. The average inhibition was 88.9 ± 2.0%. Curve 2: Inhibition by 0.039 mM NADPH without ADP. The average inhibition was 85.1 ± 4.6%. Curves 3 and 4: Inhibition in the presence of 0.2 mM ADP^{3-} by 0.039 mM NADH (curve 3) and 0.039 mM NADPH (curve 4). (B) Double-reciprocal plot of initial velocity vs. [substrate]^n, where n was the Hill coefficient for magnesium isocitrate in the presence of 0.2 mM ADP^{3-}. Curve 1, no inhibitor, n = 1.10 ± 0.07; curve 2, 0.039 mM NADH, n = 1.9 ± 0.05; curve 3, 0.039 mM NADPH, n = 1.6 ± 0.07.

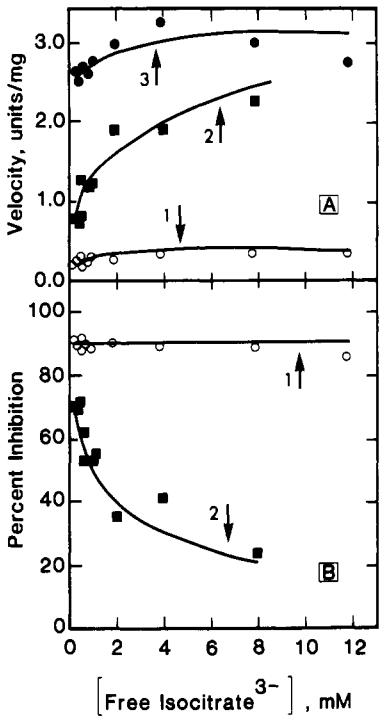

FIGURE 3: Effect of free isocitrate concentration on NAD(P)H inhibition. Reaction mixtures contained 0.25 mM NAD^+, 0.2 mM magnesium DL-isocitrate, and 0.2 mM ADP^{3-}; free isocitrate concentration varied. Curve 1, 0.039 mM NADH; curve 2, 0.039 mM NADPH; curve 3, no NAD(P)H. (A) Initial velocity vs. free isocitrate concentration. (B) Percent inhibition at each concentration of free isocitrate calculated from the data in (A).

by free isocitrate either with ADP (Figure 3, curve 2) or without ADP (not shown).

At low constant concentrations of free isocitrate (0.45 mM) and magnesium isocitrate (0.2 mM), NADH inhibition was not competitive with the cosubstrate NAD^+. Double-reciprocal plots of initial velocity vs. NAD^+ concentration shown in Figure 4A (with ADP) and Figure 4B (without ADP) were characteristic of mixed inhibition. Dixon plots of the data from Figure 1A were linear (inserts, Figure 4A,B), indicating linear mixed inhibition where the enzyme–NAD^+–NADH complex is inactive. Under these conditions, the value of α as calculated from k_{ii}/k_{is} was 4.0. However, as the concentration of free isocitrate was increased from 0.45 to 10.0 mM (experiments similar to that shown in Figure 4A), the value of α increased from 4.0 to 11.5. This may indicate a change in the mode of inhibition from the linear mixed to the linear competitive type. Thus, at higher isocitrate concentrations, the enzyme–NAD^+–NADH complex appears to become more readily dissociable. It has been reported that inhibition by NADH is competitive with respect to NAD^+ (Chen & Plaut, 1963). The earlier studies, done with relatively high concentrations of isocitrate and Mn^{2+}, seem consistent with the present finding. With NAD^+ concentration varied and isocitrate(3–) concentration fixed between 0.45 and 10.0 mM, the average K_i for NADH was 4.2 ± 0.6 μM in the presence of ADP when calculated with the aid of the computer program of Cleland (1963) for mixed inhibition. This value of K_i for NADH was comparable to that obtained when the concentrations of isocitrate and NAD^+ were constant and NADH concentration was varied (Figure 1 and Table I). NADPH inhibition with respect to NAD^+ was of the linear mixed type at low isocitrate concentration in the presence of ADP (Figure 4A, K_i = 12.6 ± 2.4 μM, α = 6.4). However, in the experiment without ADP, the data gave a better statistical fit to the equation for competitive inhibition (Figure 4B, K_i = 9.0 ± 0.7 μM).

Effect of Citrate on NAD(P)H Inhibition. Magnesium citrate with ADP or calcium citrate without ADP activated NAD-dependent isocitrate dehydrogenase from bovine heart (Plaut, 1981; Gabriel & Plaut, 1983). When ADP was present, magnesium citrate completely reversed NADPH inhibition but did not affect inhibition by NADH (Figure 5A). Without ADP, magnesium citrate did not have an effect on inhibition by either NADH or NADPH (Figure 5B). With ADP, the reversal of NADPH inhibition by magnesium citrate was not enhanced by the further presence of calcium citrate; without ADP, the combination of magnesium citrate and calcium citrate did not affect inhibition by NADPH (Table II).

Discussion

NADH and NADPH were potent inhibitors of the initial rates of NAD-specific isocitrate dehydrogenase. A number of ligands known to affect the activity of the enzyme exhibited different effects when the enzyme was inhibited by the reduced pyridine nucleotides (Table II). This was not unexpected, since fluorescence binding measurements had shown that NADH and NADPH are bound to different sites on the enzyme (Harvey et al., 1972). Thus, NADH inhibition was only reversed by magnesium isocitrate or by NAD^+. ADP was required for the competitive reversal of NADH inhibition by magnesium isocitrate, and, at subsaturating magnesium iso-

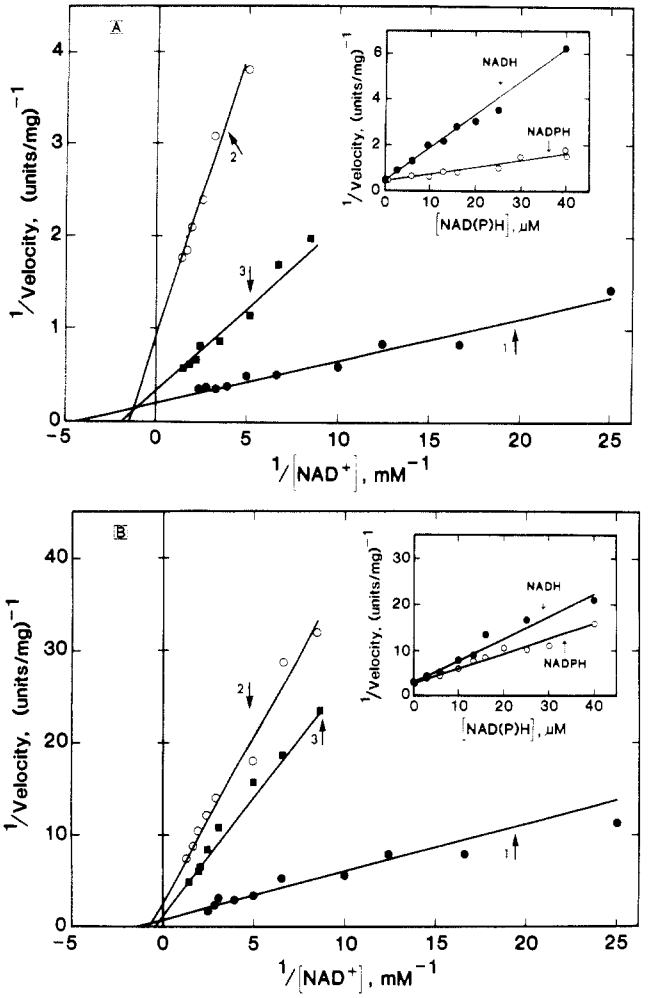

FIGURE 4: Effect of NAD^+ concentration of NADH and NADPH inhibition. Reaction mixtures contained 0.2 mM magnesium DL-isocitrate and 0.45 mM free DL-isocitrate(3−); NAD^+ concentration varied. Curve 1, no NAD(P)H; Curve 2, 0.039 mM NADH; curve 3, 0.039 mM NADPH. (A) With 0.2 mM ADP^{3-}. Insert: Dixon plot of data in Figure 1 where ADP was present. (B) No ADP. Insert: Dixon plot of data in Figure 1 where ADP was absent.

Table II: Reversal of Reduced Pyridine Nucleotide Inhibition[a]

varied ligand	$[ADP^{3-}]_{free}$ (mM)	NADH inhibition	NADPH inhibition
MI	0.0	noncompetitive	noncompetitive
MI	0.2	competitive	competitive
$Ca^{2+}{}_{free}$	0.0	no effect on inhibition	no effect on inhibition
$Ca^{2+}{}_{free}$	0.2	noncompetitive	mixed
MC	0.0	noncompetitive	noncompetitive
MC	0.2	noncompetitive	competitive
CaC-MC	0.0	no effect on inhibition	no effect on inhibition
CaC-MC	0.2	no effect on inhibition	no effect on inhibition[b]
$Ic^{3-}{}_{free}$	0.0	noncompetitive	competitive
$Ic^{3-}{}_{free}$	0.2	noncompetitive	competitive
NAD^+	0.0	linear mixed	competitive
NAD^+	0.2	linear mixed	linear mixed

[a] Reaction mixture contained 0.25 mM NAD^+, 0.2 mM magnesium DL-isocitrate, and 0.45 mM free isocitrate(3−) when not varied. The concentration of NADH or NADPH was 0.039 mM. Pyridine nucleotide inhibition is described as competitive, noncompetitive, or mixed with respect to the varied ligand. [b] No change in inhibition by CaC over the decrease in inhibition by MC.

citrate concentrations, the inhibition by NADH was of the linear mixed type with respect to NAD^+. On the other hand,

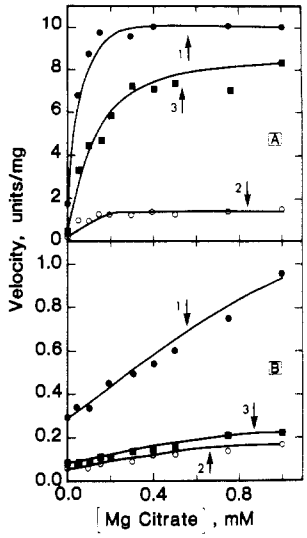

FIGURE 5: Effect of magnesium citrate concentration on NADH and NADPH inhibition. Reaction mixtures contained 0.25 mM NAD^+, 0.2 mM magnesium DL-isocitrate, and 0.45 mM free isocitrate(3−); magnesium citrate concentration varied. Curve 1, no inhibitor; curve 2, 0.039 mM NADH; Curve 3, 0.039 mM NADPH. (A) With 0.2 mM ADP^{3-}. NADH inhibition remained constant at 86.6 ± 1.3% while NADPH inhibition was reversed. (B) ADP was absent. NADH and NADPH inhibition remained constant at 80.0 ± 2.8 and 74.0 ± 1.8%, respectively.

the inhibition of NADPH was completely reversed by free isocitrate with or without ADP, it was competitive with respect to magnesium isocitrate or magnesium citrate in the presence of ADP, and it was either completely or partially reversed by NAD^+ depending on whether ADP was present or absent.

Several modulators of NAD-isocitrate dehydrogenase from mammalian tissues have been identified. The activators of the enzyme include the substrates magnesium isocitrate and NAD^+, the allosteric modifier ADP^{3-} (Chen & Plaut, 1963), Ca^{2+} (Denton et al., 1978; Aogaichi et al., 1980), and the magnesium and calcium complexes of citrate (Plaut, 1981; Gabriel & Plaut, 1983). Inhibition of the enzyme has been reported for NADH, NADPH, ATP (Chen & Plaut, 1963), and high concentrations of free Mg^{2+} (Plaut et al., 1973). The question arises as to which of the activators and inhibitors are of significance for isocitrate oxidation under physiological conditions. A partial answer can be deduced from an estimation of the concentrations of the putative modulators in the mitochondrial matrix. The calculated intramitochondrial concentrations of compounds which might affect the activity of NAD-isocitrate dehydrogenase are shown in Table III. The values are based on the ranges of concentrations of total ligands reported for isolated rat heart and rabbit heart mitochondria in state 4 to state 3 transition (LaNoue et al., 1972; Hansford & Johnson, 1975); the concentrations of free and magnesium-chelated forms of the ligands were calculated by assuming a concentration of free Mg^{2+} of 1 mM, a value within the range reported for a number of organs by Veloso et al. (1973). When the ligand concentrations in Table III are compared to kinetic constants reported previously for the bovine heart enzyme at pH 7.4 (Gabriel & Plaut, 1984), the matrix concentration of magnesium D-isocitrate should be within the regulatory range, since $S_{0.5}$ for magnesium D-isocitrate was 0.15–0.10 mM in the presence of 0.2–0.5 mM ADP. The concentrations of the cosubstrate NAD^+ (2.4–5.5 mM for total NAD^+ concentration) were much larger than $S_{0.5}$(app) for NAD^+ (0.15–0.30 mM). However, NAD^+ may also function in reversal of NADH or NADPH inhibition, and such an effect was observed (Figure 4 and Table II). A number of

Table III: Estimated Intramitochondrial Concentrations of Free and Magnesium-Chelated Forms of Certain Compounds Which Affect NAD-isocitrate Dehydrogenase Activity[a]

compound	state 4 (mM)	state 3 (mM)
D-isocitrate[b]		
free	0.14	0.03
Mg chelate	0.08	0.02
citrate[b]		
free	0.49	0.56
Mg chelate	0.86	0.99
ADP[c]		
free	0.34	2.25
Mg chelate	0.46	3.05
ATP[c]		
free	0.79	0.35
Mg chelate	7.01	3.15
NAD^+[a]		
free	2.11	5.06
Mg chelate	0.19	0.44
NADPH[b]		
free	1.06	0.99
Mg chelate	0.47	0.44

[a] The methods for calculating the free and chelated ligand concentrations and the magnesium stability constants at pH 7.4 have been described under Experimental Procedures. In addition, a value of 8.88 mM^{-1} was used for the Mg-ATP stability constants (Sillen & Martell, 1971); the magnesium stability constants of NAD^+ and NADPH were 0.088 and 0.450 mM^{-1}, respectively, as determined by the method of Gabriel et al. (1983). The concentration of free Mg^{2+} was assumed to be 1 mM. [b] Values for the total compound from rabbit heart mitochondria (Hansford & Johnson, 1975). [c] Values for the total compound from rat heart mitochondria (LaNoue et al., 1972).

investigators have reported that cellular NADH and $NADP^+$ are predominantly protein bound, whereas NAD^+ and NADPH are mostly in the free state (Chance & Baltischeffsky, 1958; Sies et al., 1972; Tischler et al., 1977). Thus, NADPH may be a more significant negative regulator of NAD-isocitrate dehydrogenase in mitochondria than NADH. In state 4 to state 3 transitions in heart mitochondria, $NADH/NAD^+$ ratios declined from 1.5 to 0.18, respectively, while the concentration of NADPH remained relatively constant at around 1.4 mM (78–98% of total NADP concentration) (Hansford & Johnson, 1975). Ignoring the effects of other modifiers, if reversal of NADPH inhibition by NAD^+ in state 4 to state 3 transitions is important for regulation of isocitrate oxidation, it would be mainly due to increasing NAD^+ concentrations. In either metabolic state, the intramitochondrial concentration of NADPH is, however, substantially larger than the value of K_i for NADPH determined under the experimental conditions shown (Table I). Likewise, the matrix concentration of magnesium citrate (Table III) is much larger than its $S_{0.5}$ value of 0.02 mM (in the presence of ADP) for enzyme activation (Gabriel & Plaut, 1984). The fact that inhibition by NADPH was reversed completely by magnesium citrate in the presence of ADP (Table II and Figure 5) suggests the possibility that the activity of the enzyme in mitochondria is determined, in part, by the poise between NADPH inhibition and magnesium citrate activation. Calcium citrate, which activated the enzyme without ADP (Gabriel & Plaut, 1983), is probably not present in mitochondria in high enough concentration even at 0.1 mM free Ca^{2+} (calculation not shown) to cause appreciable enzyme activation and did not reverse the inhibition by NADPH (Table II).

Free ADP^{3-} was the allosteric activator of purified NAD-isocitrate dehydrogenase from ox heart (Plaut et al., 1974) and porcine heart (Ehrlich & Colman, 1981). At an $S_{0.5}$(app) for ADP^{3-} of about 0.24 mM (Gabriel & Plaut, 1984), the intramitochondrial free ADP concentrations (Table III) appear to be adequate for activation and, possibly, regulation of the activity of the enzyme during state 4 to state 3 transitions. It is still controversial whether the mediation of ADP activation of NAD-isocitrate dehydrogenase by Ca^{2+} has physiological significance. Denton et al. (1978) and McCormack & Denton (1980) reported a K_m for free Ca^{2+} of 1–1.6 μM for the activation of several mitochondrial enzymes, i.e., NAD-isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase. Furthermore, it was shown that the addition of Ca^{2+} caused activation of oxidation of pyruvate and α-ketoglutarate with calcium-depleted mitochondria from brown adipose tissue (McCormack & Denton, 1980) and rat heart (Denton et al., 1980; Hansford & Cohen, 1978; Hansford & Castro, 1981). Denton and co-workers have proposed that variations in intramitochondrial free Ca^{2+} concentrations around 1 μM Ca^{2+} regulate pyruvate and citric acid cycle oxidations at the levels of these dehydrogenases. However, direct measurements of intramitochondrial free Ca^{2+} concentrations in the range of 60–150 μM in hepatocytes (Williamson & Murphy, 1981) have made it doubtful whether a meaningful control of enzyme activity can be achieved at such relatively high concentrations of matrix-free Ca^{2+} (Williamson & Cooper, 1980).[3]

The present studies suggest modes of regulation of NAD-isocitrate dehydrogenase in addition to inhibition by NADH or activation by the Ca^{2+}-facilitated lowering of the K_m of the substrate magnesium isocitrate by ADP. Thus, the activity of the enzyme may be regulated by the antagonism between positive and negative modifiers of the enzyme, with ADP functioning to regulate the other effectors.

This possibility becomes particularly apparent from the ADP requirement for the reversal of NADPH inhibition by a number of activators (Table II) and the Ca^{2+}-modulated effect of ADP concentration on the inhibition constant for NADPH (Table I). In the absence of complete knowledge of the relevant kinetic constants and the intramitochondrial concentrations of the critical ligand species, it is difficult at present to document such a complex mechanism of regulation of the enzyme. However, there is some support for such a model. For example, in an experiment in which the concentrations of NAD^+ (0.25 mM), magnesium DL-isocitrate (0.20 mM), NADPH (0.039 mM), and magnesium citrate (1.0 mM) were held constant, the addition of 0.20 mM ADP^{3-} increased the initial velocity 40-fold (from 0.22 to 8.42 units/mg). Under the same conditions but without NADPH, the addition of ADP increased the rate only 10-fold (from 0.95 to 9.93 units/mg).

In such a regulatory mechanism, the concentration of Ca^{2+} required for ADP-mediated reversal of the action of a negative modulator may also differ from that needed for activation of the enzyme by ADP without the inhibitor. This is supported by the effect of varying concentrations of Ca^{2+} on the reversal of NADPH inhibition by ADP (conditions similar to that shown in Table I). The $S_{0.5}$ for Ca^{2+} was 48 ± 11 μM. This value was nearly 80 times larger than the $S_{0.5}$ for Ca^{2+} (0.6 μM) for activation of the enzyme, at equal concentrations of ADP and magnesium isocitrate but in the absence of NADPH (Aogaichi et al., 1980). Thus, the concentration of Ca^{2+} required for reversal of NADPH inhibition is much closer to the range of intramitochondrial free Ca^{2+} levels reported by

[3] Even the more recent values of intramitochondrial free $[Ca^{2+}]$ of 9–13 μM for rat liver and 4–6 μM for rat heart (Coll et al., 1982) are substantially larger than the K_m for free Ca^{2+} of 0.3–1 μM for the activation of NAD-isocitrate dehydrogenase (Denton et al., 1978; Aogaichi et al., 1980).

Williamson & Murphy (1981) and Coll et al. (1982) than the $S_{0.5}$ for Ca^{2+} for enzyme activation without the inhibitor.

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