Kinetic Regulation of Yeast NAD-Specific Isocitrate Dehydrogenase by Citrate

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ABSTRACT: The present results suggest that the enzyme modifier citrate and the substrate isocitrate are bound at different sites on yeast NAD-specific isocitrate dehydrogenase and that citrate diminishes the binding of the positive effector 5′-AMP, thereby causing a decreased rate of enzyme catalysis. This interpretation differs from the earlier proposal that citrate can replace isocitrate at an activator site on the enzyme and can cause inhibition by binding at its catalytic site [Atkinson et al. (1965) *J. Biol. Chem. 240*, 2682]. The present proposal is supported by the following observations: At constant subsaturating levels of isocitrate, NAD^+, and Mg^{2+} without AMP, up to 10 mM citrate was an activator and not an inhibitor. Citrate decreased velocity for AMP-activated enzyme; however, with increasing citrate the specific activity with AMP asymptotically approached but did not decrease below the level of the enzyme maximally activated by citrate in the absence of AMP. When added singly, AMP decreased $S_{0.5}$ for isocitrate without changing the Hill number (n), whereas citrate lowered n without changing $S_{0.5}$ for isocitrate. The difference in action of these modifiers indicated that they were bound at separate sites on the enzyme. The binding of citrate appeared to cause a conformational change in the protein that lowered the enzyme's affinity for AMP. This was consistent with the findings that citrate (or the citrate agonist fluorocitrate) (i) resulted in an increase in $S_{0.5}$ for isocitrate with the AMP-activated enzyme and (ii) decreased binding of the positive effector analogue TNP-AMP as measured by fluorescence change. Double inhibitor and other kinetic experiments with citrate and the isocitrate antagonist *threo*-α-methylisocitrate have demonstrated that the binding sites on the enzyme for citrate and isocitrate are mutually independent of one another.

Adenine nucleotides and citrate are modulators of most NAD-specific isocitrate dehydrogenases. The adenine nucleotides activate by lowering the apparent K_m for substrate without affecting maximal velocity, whereas the nature of the kinetic effects of citrate varies considerably depending on the source of the enzyme. Thus, without AMP citrate activated the enzyme from bakers' yeast and lowered the Hill number for isocitrate, suggesting citrate binding at a regulatory site, whereas, when activated by AMP, inhibition by citrate competitive with respect to isocitrate was observed. The latter has been interpreted as citrate binding to the catalytic isocitrate-binding sites (Atkinson et al., 1965). In contrast, citrate was not an inhibitor either with or without the adenine nucleotide effector ADP for the bovine heart enzyme. The heart enzyme was activated by citrate without changing the Hill number of isocitrate in the absence of ADP, and, in fact, the presence of ADP facilitated citrate activation by lowering the $S_{0.5}$ for citrate. This indicated that citrate did not replace isocitrate at either catalytic or regulatory binding sites on the heart enzyme (Gabriel & Plaut, 1984).

The magnesium chelates of isocitrate and citrate were the active substrate and activator species for the bovine heart enzyme (Plaut et al., 1974; Gabriel & Plaut, 1984), whereas free Mg^{2+} and free isocitrate were required for the activity of the yeast enzyme (Gabriel & Plaut, 1986). It seemed possible that some of the differences of the enzymes from bovine heart and yeast were due to metal chelation resulting in inappropriate changes in free or metal-chelated forms of substrates and activators. When the concentrations of free Mg^{2+} and free and metal-chelated ligands were maintained at defined levels in the present experiments with the yeast enzyme, citrate was an activator without AMP and an inhibitor in the presence of AMP. However, under these conditions, the specific activity of the enzyme maximally activated by citrate alone was smaller than that activated by AMP alone. Several lines of evidence are presented here that citrate diminishes the binding of AMP, resulting in a species of the enzyme with a lowered affinity for isocitrate and, hence, decreased catalytic efficiency. The present experiments exclude binding of citrate and isocitrate to common catalytic or activator sites on the yeast enzyme. Preliminary reports of this work have appeared (Gabriel & Plaut, 1989; Plaut & Gabriel, 1989).

Experimental Procedures

Materials. DL-*threo*-Isocitric acid lactone purchased from Aldrich Chemical Co. (Milwaukee, WI) was recrystallized from ethyl butyrate and hydrolyzed as previously described (Gabriel & Plaut, 1980). The ligand concentrations are reported on the basis of the DL-isomer in the reaction mixture, even though the enzyme is stereospecific for D-*threo*-isocitrate. NAD^+ and 5′-AMP were obtained from Boehringer-Mannheim (Indianapolis, IN). NAD^+ was purified by reversed-phase HPLC to remove a small but kinetically significant contamination of 5′-AMP as previously described (Gabriel & Plaut, 1990). TNP-AMP[1] was obtained from Molecular Probes (Eugene, OR) and was purified by chromatography on Whatman 3MM paper (Gabriel & Plaut, 1990).

Fluorocitric acid purchased from Sigma (St. Louis, MO) predominantly contained the DL-erythro diastereoisomeric pair as determined by high-voltage paper electrophoresis (Fanshier et al., 1964). The hydroxycitrate (1,2-dihydroxypropane-1,2,3-tricarboxylate) diastereoisomers DL-hibiscus acid lactone

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[1] Abbreviations: TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-monophosphate; α-methylisocitrate, DL-*threo*-α-methylisocitrate.

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(1*R**,2*S** isomer pair) and DL-garcinia acid lactone (1*R**,2*R** isomer pair) were synthesized as previously described (Plaut et al., 1975a). L-Hibiscus acid lactone and L-garcinia acid lactone were isolated from *Garcinia cambogia* and *Hibiscus sabdariffa*, respectively (Lewis, 1969). Synthetic procedures for the preparation of DL-*threo*-α-methylisocitric acid (Plaut et al., 1975b), β-mercapto-α-ketoglutarate (Plaut et al., 1986), and DL-homocitric acid and *O*-methyl citrate (Gabriel et al., 1983) have been reported previously.

Other chemicals used were reagent grade and were obtained commercially.

Kinetics. NAD^+-specific isocitrate dehydrogenase was purified from bakers' yeast and stored as described previously (Gabriel & Plaut, 1990). Velocities were determined by measuring the formation of NADH spectrophotometrically at 340 nm in a medium containing free 0.5 mM Mg^{2+}, 0.15 mM free DL-*threo*-isocitrate, 0.33 mM NAD^+, 50 mM Tris–acetate at pH 7.4, 1 mg/mL bovine serum albumin, and purified enzyme at a final concentration of 2×10^{-4} mg/mL. Deviations from the above medium composition and, when present, the concentrations of 5′-AMP, citrate, and other modifiers have been indicated in the table and figure legends. Reactions were initiated by the addition of enzyme except that they were started with NAD^+ when β-mercapto-α-ketoglutarate was tested. Initial 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/min at 25 °C. Kinetic parameters were calculated from initial velocities by fitting the data to the appropriate computer programs developed by Cleland (1963).

In the present study, the concentrations of free isocitrate and free Mg^{2+} were maintained at the desired levels since it has been established that free isocitrate is the substrate and free Mg^{2+} the activator of this enzyme (Gabriel & Plaut, 1986). Cennamo et al. (1967) reported that the activity of the enzyme was inhibited by high ionic strength and that there were qualitative differences between anions in affecting enzyme activity. Thus, sulfate, phosphate, and chloride were appreciably more inhibitory than Hepes or acetate. The effects of high ionic strength and inhibitory anions could be reversed by AMP. We have confirmed these results, and in order to minimize these effects in the current experiments, acetate was used as the only counterion and ionic strength was maintained close to 0.05 M in all assays reported here.

The concentrations of the free and metal-chelated forms of the ligands used were maintained at the levels indicated in the text, tables, and figures by adjusting the total ligand and magnesium concentrations as described previously (Gabriel et al., 1986). Stability constants at pH 7.4 for the magnesium carboxylates examined are given in Table II.

Spectroscopy. Fluorescence spectra were measured in 0.1-mL volume, 0.32-cm path-length cells with a Perkin-Elmer Model 650-10S fluorometer. All measurements were corrected for fluctuations in the exciting lamp intensity. The concentration of TNP-AMP was determined spectroscopically in 0.2 M Tris-HCl at pH 8.0 by using a molar extinction coefficient of 26.0×10^3 M^{-1} cm^{-1} (Hiratsuka, 1982). Enzyme samples were prepared as previously described (Gabriel & Plaut, 1990).

Results

Modulation of Enzyme Activity by Citrate

Kinetic Effects. Hathaway and Atkinson (1963) reported that yeast NAD-isocitrate dehydrogenase was activated by low levels of citrate when isocitrate, NAD^+, and Mg^{2+} were saturating and AMP was absent; citrate inhibited when the concentration of the positive effector AMP was saturating. The activation by citrate without AMP resulted in a decrease of the Hill number but not the $S_{0.5}$ for total isocitrate (Atkinson et al., 1965). While the trends in effects of citrate had been indicated in the earlier experiments from Atkinson's laboratory, no data on changes of the specific activity of the enzyme with varying citrate were reported.

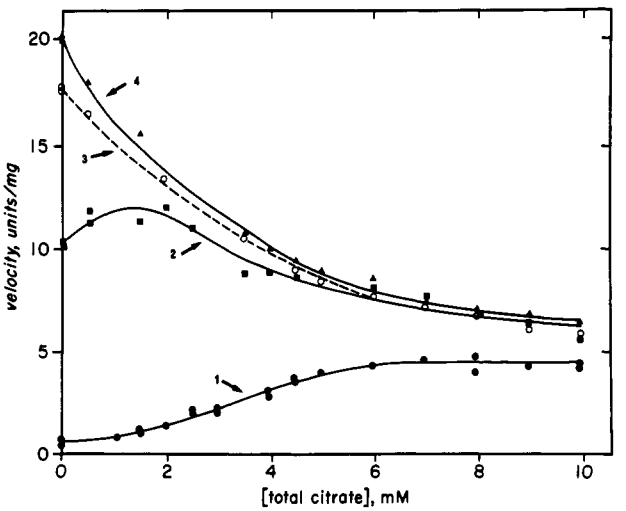

FIGURE 1: Effect of citrate on enzyme activity at different concentrations of AMP. The AMP concentrations used were curve 1, none (●); curve 2, 0.01 mM (■); curve 3, 0.5 mM (○); and curve 4, 1.5 mM (▲). The composition of the reaction mixtures was as reported under Experimental Procedures except that the concentration of free Mg^{2+} was 0.15 mM.

When the concentrations of free isocitrate, free Mg^{2+}, and NAD^+ were kept constant without AMP and total citrate was varied, activity increased up to about 6 mM citrate and then became constant up to 10 mM citrate (curve 1, Figure 1). The results parallel those reported by Hathaway and Atkinson (1963) except that they noted decreased activity at 7 mM citrate [Figure 12A in Hathaway and Atkinson (1963)]. The latter was probably due to chelation of divalent cation by citrate since recalculation of the data suggests that the concentration of activating free Mg^{2+} may have become limiting at their ligand concentrations. Without citrate, the specific activity with 0.5 and 1.5 mM AMP was about 4 times larger, and with 10 μM AMP the specific activity was about twice as large, as with the highest concentrations of citrate without AMP (curves 2–4 vs 1, Figure 1). In agreement with Hathaway and Atkinson (1963), inhibition by citrate of the AMP-activated enzyme was found; however, with increasing citrate the specific activity with AMP asymptotically approached but did not fall below the maximal activation without AMP (Figure 1).

Kinetic constants from a series of experiments under the conditions shown in Figure 2 and from the Hill plots shown in the insert of Figure 2 have been summarized in Table I. Without AMP, the addition of citrate resulted in a lowering of the Hill number for isocitrate without affecting $S_{0.5}$ for free isocitrate. Without citrate, AMP did not lower the Hill number for isocitrate but markedly decreased the $S_{0.5}$ for free isocitrate. When both citrate and AMP were present, the Hill number for isocitrate was decreased as with citrate alone, but the $S_{0.5}$ for isocitrate was appreciably higher than with AMP alone. The effect of citrate on the action of AMP suggested that binding of citrate changed the enzyme to a form with a decreased affinity for AMP. This possibility was examined by testing the effect of citrate on the binding to the enzyme

Table I: Effect of Citrate on Kinetic Constants for Isocitrate[a]

[citrate] (mM)	[AMP] (mM)	isocitrate		
		Hill no. (n)	$S_{0.5}$ (mM)	V (units/mg)
0.0	0.0	2.4 ± 0.12 (43)	1.42 ± 0.07 (47)	21.8 ± 0.9 (47)
10.0	0.0	1.2 ± 0.2 (12)	1.53 ± 0.1 (12)	24.9 ± 1.2 (12)
0.0	0.2	1.9 ± 0.09 (34)	0.09 ± 0.004 (41)	19.8 ± 0.5 (41)
10.0	0.2	1.2 ± 0.02 (29)	0.36 ± 0.02 (29)	20.8 ± 0.6 (29)

[a] The composition of reaction mixtures was as described under Experimental Procedures except that free magnesium was 0.15 mM and isocitrate was varied. Citrate and AMP were present as indicated. The number of data points is shown in parentheses.

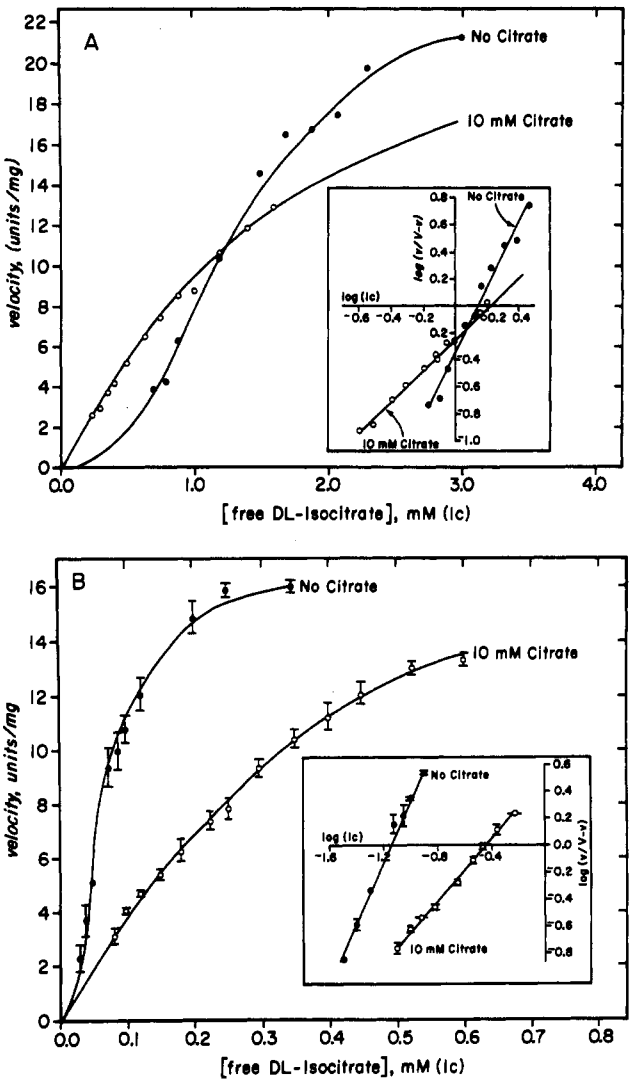

FIGURE 2: Effect of citrate on the $S_{0.5}$ for isocitrate. The conditions of incubation were as under Experimental Procedures except that free Mg^{2+} was 0.15 mM. Citrate was either absent (●) or present at 10 mM (○). (A) No AMP; (B) with 0.2 mM AMP. Insert: Hill plot of the data.

of the fluorescent analogue TNP-AMP.

Effect of Citrate on AMP Binding. A number of analogues of AMP were active positive modifiers of yeast NAD-isocitrate dehydrogenase (Gabriel & Plaut, 1990). The fluorescent analogue TNP-AMP (Hiratsuka, 1982), which activated the enzyme nearly as well as AMP, showed an enhancement of fluorescence intensity that was accompanied by a shift from 546 to 536 nm upon binding to the enzyme (Gabriel & Plaut, 1990). If citrate were indeed to diminish AMP binding to the enzyme, this could be reflected in a spectral change of the combination of TNP-AMP and the ezyme when compared with and without citrate. This was found to be the case since the fluorescence enhancement of TNP-AMP in the presence of enzyme was decreased by 43%, i.e., it decreased from 3.5-

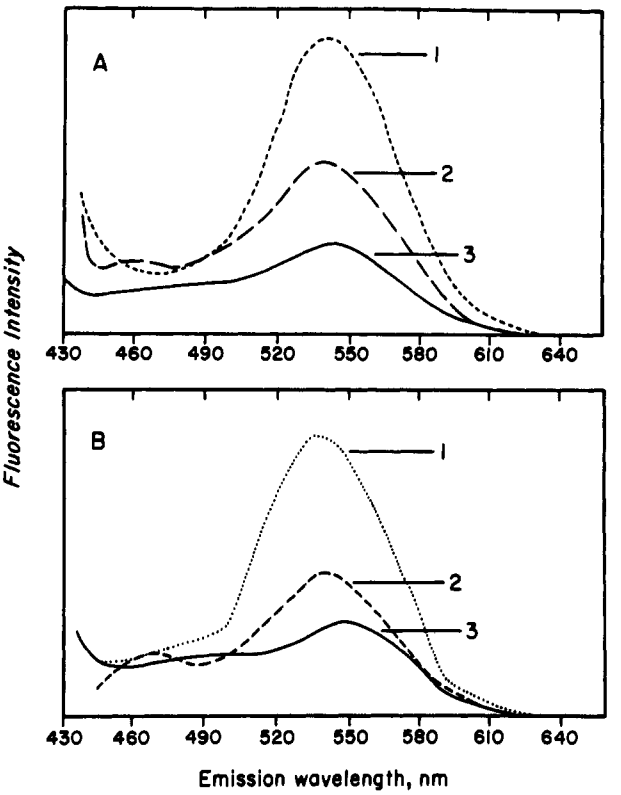

FIGURE 3: Effect of tricarboxylates on the fluorescence emission spectra of TNP-AMP. The excitation wavelength was 410 nm. Sample composition included 10.5 μM TNP-AMP, 1.7 μM yeast enzyme (octomer), 10 mM tricarboxylate, and 50 mM Tris–acetate at pH 7.4. The appropriate buffer and free enzyme spectra were subtracted out. Curve 1, enzyme plus TNP-AMP; curve 2, enzyme plus TNP-AMP plus tricarboxylate; curve 3, TNP-AMP alone. The tricarboxylates tested were citrate (panel A) and fluorocitrate (panel B).

fold enhancement without citrate (curve 1, Figure 3A) to a 2-fold enhancement with citrate (curve 2, Figure 3A). These experiments were repeated with DL-*erythro*-fluorocitrate, which is an even more effective modifier than citrate of yeast NAD-isocitrate dehydrogenase either as an activator without AMP or as an inhibitor with AMP (Table II). These kinetic effects paralleled the present finding that fluorocitrate also decreased the fluorescent enhancement of TNP-AMP more (51%) than the equivalent concentration of citrate (Figure 3B). The addition of either citrate or fluorocitrate to the combination of enzyme and TNP-AMP resulted in the expected red shift of the fluorescence emission of the enzyme-bound probe to that of free TNP-AMP (cf. curves 1–3, Figure 3B).

In experiments previously reported (Gabriel & Plaut, 1990), the fluorescence intensity of the enzyme–TNP-AMP complex was not diminished by magnesium ion and isocitrate, although the further addition of 5′-AMP did result in decreased fluorescence enhancement. The latter indicated that TNP-AMP and AMP may compete at the same site on the dehydrogenase. The studies on the effects of citrate and fluo-

Table II: Effectiveness of Carboxylates as Modulators of Enzyme Activity[a]

compound	stability constants[b] (mM^{-1})	no AMP		0.2 mM AMP	
		action[c]	activation (fold) or inhibition (%)	action[c]	activation (fold) or inhibition (%)
tricarboxylates					
citrate	1.78	A	7.7	I	40
DL-*erythro*-fluorocitrate	0.538	A	11.2	I	78
DL-hibiscusate	0.912	A	5.2	I	89
L-hibiscusate	0.912	A	3.5	I	86
L-garciniate	0.325	I	100	I	58
DL-*threo*-α-methylisocitrate	0.440	I	100	I	100
O-methyl citrate	4.26	NE		NE	
DL-homocitrate	0.642	I	100	NE	
dicarboxylates					
fumarate	0.01	I	100	NE	
DL-malate	0.05	I	100	NE	
D-malate	0.05	I	100	NE	
L-malate	0.05	I	100	NE	
maleate	0.055	I	78	NE	
malonate	0.129	I	95	NE	
succinate	0.015	I	85	NE	
D-tartrate	0.023	I	76	NE	
L-tartrate	0.023	I	76	NE	
β-mercapto-α-ketoglutarate		I	100	I	100
monocarboxylates					
glycolate	0.008	NE		NE	
L-lactate	0.009	NE		NE	

[a] The composition of assay mixtures was as described under Experimental Procedures. The analogues were tested at a concentration of 10 mM. Velocities without added analogues were 0.134 ± 0.4 unit/mg (14 data points) and 18.96 ± 0.81 units/mg (6 data points) without AMP and with 0.2 mM AMP, respectively. [b] Magnesium chelate at pH 7.4. [c] Abbreviations: A, activator; I, inhibitor; NE, no effect.

rocitrate on fluorescence enhancement in Figure 3 were done without added activating divalent metal ions, suggesting that unchelated citrate is the active form of the effector. When fluorocitrate was present (curve 2, Figure 3B), the further addition of 3 mM isocitrate alone or 14 mM isocitrate plus 4.5 mM magnesium acetate did not significantly alter the fluorescence emission spectrum (data not shown), indicating that these ligands did not displace fluorocitrate from the enzyme since, under such circumstances, a rebinding of TNP-AMP might be expected.

Effects of Other Carboxylates

The specificity of features of the chemical structure of citrate as a modulator of yeast NAD-isocitrate dehydrogenase was examined by testing a number or tri-, di-, and monocarboxylates for substrate, activator, and inhibitor activity.

Classification of Modulators. The kinetic effects of the compounds tested and the stability constants of their magnesium chelates have been summarized in Table II. When the compounds were examined without isocitrate, only D-garciniate [(1*R*,2*R*)-1,2-dihydroxy-1,2,3-propanetricarboxylate] was a substrate for the enzyme. The other compounds could be grouped into three classes (classes I–III) depending on whether they activated, inhibited, or had no effect with or without AMP (Table III). Among the tricarboxylates, DL-*erythro*-fluorocitrate and the hydroxycitrate diastereoisomers with the hydroxyl groups at the α and β positions in the threo configuration had the same activity as citrate (class I). Other tricarboxylates fell either into class II (homocitrate) or into class III (α-methylisocitrate) or were inactive (*O*-methyl citrate). Most dicarboxylates belonged to class II; i.e., they were inhibitors in the absence of AMP and had no effect with AMP. α-Methylisocitrate and β-mercapto-α-ketoglutarate were potent inhibitors competitive with respect to isocitrate, oxalosuccinate, or α-ketoglutarate for NADP:isocitrate dehydrogenase from heart but showed no or little inhibition of the NAD-specific enzyme from animal sources (Plaut et al., 1975b, 1986; Kelly & Plaut, 1981). However, these compounds were effective inhibitors of yeast NAD:isocitrate dehydrogenase either with or without AMP (class III).

Table III: Classification of Compounds by Modulator Activity[a]

class	effect		compound
	no AMP	0.2 mM AMP	
I	activation	inhibition	citrate
			fluorocitrate
			DL-hibiscusate
			L-hibiscusate
II	inhibition	no effect	fumarate
			homocitrate
			D-malate
			L-malate
			maleate
			malonate
			succinate
			D-tartrate
			L-tartrate
III	inhibition	inhibition	L-garciniate
			β-mercapto-α-ketoglutarate
			DL-*threo*-α-methylisocitrate

[a] The following compounds had no effect on enzyme activity: glycolate, L-lactate, and *O*-methyl citrate.

Interactions of Modulators with Enzyme. The modification of enzyme activity by the class I effector citrate was no longer observed when isocitrate concentration approached saturation (Gabriel & Plaut, 1989). The inhibition was competitive with respect to isocitrate for the class II compound L-malate and for the class III substances α-methylisocitrate and β-mercapto-α-ketoglutarate (experiments 1, 2, and 5 in Table IV). Although the patterns of modification of enzyme activity by the compounds in the three classes of effectors were clearly different, the reversal of their effects by isocitrate might be due to their interaction with the enzyme at common isocitrate-binding sites. This possibility was examined in kinetic experiments

Interactions of Modulators. The interaction of two inhibitors binding to an enzyme can be examined by plotting v_o/v_i (where v_o is initial velocity in the absence of both inhibitors and v_i is initial velocity in the presence of one or both inhib-

Table IV: Nature and Modification of Action of Inhibitors[a]

expt	inhibitor	[DL-isocitrate] (mM)	[AMP] (mM)	[citrate] (mM)	inhibition		
					type	kinetic constant	value ± SD[b]
1	L-malate[c]	varied	0.0	0.0	comp	app K_i	4.3 ± 0.4 mM (42)
2	α-methylisocitrate	varied	0.2	0.0	comp	app K_i	0.08 ± 0.01 mM (29)
3A	α-methylisocitrate	0.15	0.2	0.0		$I_{0.5}$	0.12 ± 0.06 mM (7)
3B	α-methylisocitrate	0.15	0.2	10.0		$I_{0.5}$	0.39 ± 0.04 mM (7)
4A	α-methylisocitrate	0.15	0.0	0.0		$I_{0.5}$	3.4 ± 0.07 mM (10)
4B	α-methylisocitrate	0.15	0.2	0.0		$I_{0.5}$	0.15 ± 0.01 mM (19)
5	βSHαKG[d]	varied	0.2	0.0	comp	app K_i	0.09 ± 0.01 μM (27)
6A	βSHαKG[d]	0.15	0.2	0.0		$I_{0.5}$	0.13 ± 0.02 μM (17)
6B	βSHαKG[d]	0.15	0.2	10.0		$I_{0.5}$	5.6 ± 0.16 μM (16)

[a] The assay conditions were as described under Experimental Procedures except where noted. [b] Kinetic constants for α-methylisocitrate were based on the concentration of free analogue while those for β-mercapto-α-ketoglutarate and L-malate were calculated on the basis of total analogue concentration. The number of data points is shown in parentheses. [c] The reaction mixtures contained 2.0 mM free Mg^{2+}. [d] β-Mercapto-α-ketoglutarate.

itors) vs the concentration of one inhibitor while the concentration of the second inhibitor is held constant. This process is repeated at several concentrations of the second inhibitor. According to the criteria developed by Yonetani and Theorell (1964), the inhibitors are mutually exclusive only when a pattern of parallel lines is obtained in such plots, i.e., when the interaction coefficient α is ∞.

A Yonetani–Theorell plot of the effects of L-malate and α-methylisocitrate showed a pattern of intersecting lines. This suggests that although both compounds are inhibitors competitive with isocitrate in the absence of AMP, they interact at different sites on the enzyme. Furthermore, the value of $\alpha = 1.0$ obtained from this plot is consistent with one inhibitor not affecting the binding of the other.

The double inhibitor plot of varying α-methylisocitrate with and without citrate in the presence of AMP also exhibited intersecting lines, indicating that these tricarboxylates too interact at different sites on the enzyme. The inhibition constant for DL-*threo*-α-methylisocitrate calculated from the data in the above experiment was about 3-fold larger with than without citrate (experiments 3A and 3B in Table IV). The increase by citrate of $I_{0.5}$ for α-methylisocitrate an inhibitor mutually exclusive with respect to isocitrate, is consistent with the increase by citrate of the $S_{0.5}$ for the substrate isocitrate in the presence of AMP (Table I). The marked decrease of $I_{0.5}$ for α-methylisocitrate by AMP in the absence of citrate (experiment 4A vs 4B in Table IV) is also compatible with the lowering by AMP of $S_{0.5}$ for isocitrate under equivalent conditions (Table I).

Results similar to those with α-methylisocitrate were obtained with the substantially more potent competitive inhibitor β-mercapto-α-ketoglutarate (experiments 5 and 6 in Table IV). A pattern of intersecting lines was found in the Yonetani–Theorell plot of the double inhibitor experiment with β-mercapto-α-ketoglutarate and citrate in the presence of AMP, indicating that these carboxylates also did not compete for the same site on the dehydrogenase. As with α-methylisocitrate citrate markedly increased the value of $I_{0.5}$ in the presence of AMP (experiments 6A and 6B in Table IV).

Discussion

Hathaway and Atkinson (1963) and Atkinson et al. (1965) proposed that citrate mimicked the action of isocitrate by replacing isocitrate at an activator site on the enzyme and by causing inhibition due to binding at the catalytic site. While most of the basic observations of the Atkinson group on the effects of the modifiers AMP and citrate on the kinetic behavior of the enzyme were confirmed in the present study, additional experimental observations suggest an alternate mechanism of modulation of enzyme activity by citrate that does not involve replacement of isocitrate at either a regulatory or catalytic site. It is proposed instead that binding of citrate to a unique site on the enzyme causes a protein conformational change resulting in a form of the enzyme with (i) enhanced activity in the absence of AMP but (ii) diminished capacity for binding of the positive effector AMP. Since the relative catalytic activity of the enzyme activated by AMP is larger than that activated by citrate, the addition of citrate to the AMP-activated enzyme would result in apparent inhibition. Data consistent with this proposal are summarized below.

When the effect of increasing citrate was examined with and without AMP at subsaturating isocitrate, Mg^{2+}, and NAD^+ levels, the specific enzyme activity with AMP did not decrease below that of the maximally activated enzyme without AMP (Figure 1). This is contrary to the continuous decline in velocity expected with increasing citrate concentrations if citrate, which is not a substrate, replaced isocitrate at the catalytic site. Since the same level of specific activity was approached with increasing citrate either with or without AMP (Figure 1), a conformational change of the enzyme caused by the binding of citrate to a single site may explain the activation (without AMP) and the diminished activation by AMP. Consistent with such a possibility is the similarity of the magnitude of the kinetic constants for citrate activation without AMP ($S_{0.5}$ = 5.7 mM) and for citrate inhibition with AMP ($I_{0.5}$ = 4.9 mM) determined under the conditions of Figure 1.

The kinetic patterns obtained when isocitrate concentration was varied suggest that citrate and isocitrate do not bind to the same regulatory site on the enzyme (Figure 2 and Table I). Thus, whereas AMP caused a decrease in $S_{0.5}$ for isocitrate without a substantial change in Hill number (n) when citrate was absent (Table I), citrate added without AMP decreased n without altering $S_{0.5}$ for isocitrate (Figure 2A). If citrate and isocitrate were functionally equivalent in binding to the same regulatory site, one would have expected n to vary with isocitrate concentration. As indicated by the linearity of the Hill plots in the absence of citrate, the value of n was not affected by changing isocitrate concentrations either with or without AMP (Figure 2, inserts). Furthermore, in the absence of AMP and with isocitrate varied, citrate activated at substrate concentrations below $S_{0.5}$ for isocitrate, diminished velocity at levels of isocitrate above $S_{0.5}$, but reached the same V with or without citrate as isocitrate became saturating (Figure 2A). The effect of citrate under the conditions of Figure 2A could be simulated by fitting the averages of the relevant kinetic constants in Table I to the Hill equation without considering that citrate binds to either a catalytic or regulatory isocitrate binding site.

Both $S_{0.5}$ and n for isocitrate were changed when both citrate

and AMP were present (Figure 2B, Table I). The results suggest that citrate may decrease the activation by AMP by diminishing the binding of the nucleotide to the enzyme. This is consistent with the nature of the decrease of enzyme-specific activity with increasing citrate in the presence of AMP (Figure 1) and the finding that the value of $S_{0.5}$ for isocitrate was larger when both citrate and AMP were present than with AMP alone (Table I). This possibility receives further support by the observation that the fluorescence enhancement of the combination of enzyme and TNP-AMP was decreased by citrate (Figure 3).

Studies with analogues of citrate and isocitrate have confirmed and extended the conclusion that neither the positive nor negative modifier effects of citrate are mediated by binding to the same site(s) on the enzyme to which isocitrate is bound. Thus, the inhibition by citrate and the isocitrate antagonist α-methylisocitrate differed depending on whether or not AMP was present (Table III); double inhibitor experiments indicated that these tricarboxylates were not bound to a common site on the dehydrogenase. The value of the Hill number of isocitrate was not affected by either increasing concentrations of isocitrate (Figure 2) or α-methylisocitrate (not shown), which is contrary to the effect of citrate (Table I) and the purported binding of citrate and isocitrate at a common activator site. AMP lowered $I_{0.5}$ for α-methylisocitrate without citrate and, in the presence of AMP, citrate raised $I_{0.5}$ for α-methylisocitrate (Table IV), which paralleled the increase by citrate of $S_{0.5}$ for isocitrate (Table I). The present proposal that this kinetic effect of citrate can be attributed to its induction of a conformer of the enzyme with lowered affinity for the positive effector AMP was supported by the finding that, like citrate, the citrate agonist fluorocitrate (Tables II and III) decreased the binding to the enzyme of the active modifier TNP-AMP, as measured by changes in fluorescence characteristics (Figure 3).

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