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Evidence for Isosteric and Allosteric Nucleotide Inhibition of Citrate Synthase from Multiple-Inhibition Studies

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Citrate synthases from diverse organisms are inhibited by ATP and NADH. Evidence is presented, from multiple-inhibition studies on various citrate synthases, that ATP acts in all cases as an isosteric inhibitor at the acetyl-CoA site. On the other hand, NADH also acts isosterically with eukaryotic and Gram-positive bacterial citrate synthases, but behaves as an allosteric inhibitor specifically in the case of the Gram-negative bacterial enzyme. After desensitization to this allosteric inhibition, only the isosteric nucleotide inhibition, as found in other citrate synthases, is observed.

The regulation of the activity of citrate synthase (EC 4.1.3.7) through inhibition by various nucleotides, in particular ATP and NADH, may make an important contribution to the overall control of the citric acid cycle. Since the first demonstration by Hathaway & Atkinson (1965) of the inhibition of yeast citrate synthase by ATP, many other citrate synthases have been shown to be similarly affected (Srere, 1972; Weitzman & Danson, 1975). However, Weitzman & Jones (1968) showed that Gram-negative bacteria differ from other organisms in having citrate synthases which are specifically inhibited by NADH, and the desensitization of several of these indicates that the NADH site is distinct from the active site (Weitzman & Danson, 1975). Moreover, the citrate synthases of Gram-negative bacteria differ from those of Gram-positive bacteria and eukaryotic organisms in having a considerably higher molecular weight (Weitzman & Dunmore, 1969).

The specific inhibition by NADH in the Gram-negative bacterial group of citrate synthases has been challenged by Srere (1972, 1974) on the grounds that other citrate synthases also show some sensitivity to NADH inhibition. However, the very much higher concentrations of NADH required to produce inhibition, the non-specificity of NADH (NADPH, NAD^+ and $NADP^+$ are also inhibitory) and the failure to observe desensitization serve to differentiate the NADH inhibition of eukaryotic and Gram-positive bacterial citrate synthases from that of the Gram-negative bacterial enzymes. It is our view that the observed inhibition in the former cases arises from the structural similarity between nucleotides and the substrate acetyl-CoA; this manifests itself in a general non-specific affinity of the active site for adenine and nicotinamide–adenine nucleotides which results in inhibition of enzymic activity by all such compounds, acting isosterically (Weitzman & Danson, 1975).

The present work was undertaken in an attempt to gain experimental support for this view. We have applied the method of multiple-inhibition analysis (Yonetani & Theorell, 1964) to the nucleotide inhibition of several citrate synthases of different types. This method involves examination of the multiple inhibition of an enzyme by two inhibitors, each competitive with the same substrate. Graphical analysis of the results then indicates whether the two inhibitors act on the same or different sites of the enzyme. The results reported in the present communication provide evidence that the ATP inhibition of diverse citrate synthases and the NADH inhibition of eukaryotic and Gram-positive bacterial citrate synthases are isosteric effects exerted at the active site. In contrast, the NADH inhibition of Gram-negative bacterial citrate synthase is confirmed to be an allosteric effect.

Experimental

Pig heart citrate synthase, oxaloacetic acid, CoA and NADH were from Boehringer Corp. (London) Ltd., London W.5, U.K.; ATP and 5,5′-dithiobis-(2-nitrobenzoic acid) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were the finest grade commercially available.

Acetylation of CoA was carried out as described by Stadtman (1957). Bromoacetyl-CoA was prepared by transacylation from bromoacetylthiophenol to CoASH by the method of Chase & Tubbs (1969) and, before use, was passed through a small column of Sephadex G-10. Its concentration was determined by using it as a substrate for pig heart citrate synthase and measuring the total reaction in the presence of

excess of oxaloacetate and 5,5'-dithiobis-(2-nitrobenzoate).

Bacillus megaterium and *Pseudomonas aeruginosa* were grown aerobically in nutrient broth at 30°C. The cells were harvested and washed, and sonicated cell-free extracts were prepared in 20mM-Tris–HCl (pH8.0)–1mM-EDTA–1mM-dithiothreitol. Partially purified enzyme preparations were obtained by treating the sonicated extracts with protamine sulphate (1mg/10mg of protein), fractionation with $(NH_4)_2SO_4$, and gel filtration of the resuspended 50–70%-satd. fraction through a column of Sephadex G-200 with the same buffer system as above. *Ps. aeruginosa* citrate synthase was desensitized to NADH inhibition by treatment with 0.1mM-5,5'-dithiobis-(2-nitrobenzoate) in 20mM-Tris–HCl, pH8.0, for 20min at room temperature (20°C). The modified enzyme was separated from reagents by gel filtration on a column of Sephadex G-25.

Citrate synthase was assayed spectrophotometrically at 412nm and 25°C by the method of Srere *et al.* (1963). Reaction mixtures contained 20mM-Tris–HCl, pH8.0, 1mM-EDTA, 0.2mM-oxaloacetate and 0.1mM-5,5'-dithiobis-(2-nitrobenzoate). During enzyme purification, assays were performed with 150μM-acetyl-CoA; for multiple-inhibition studies, the acetyl-CoA concentration was decreased to 15μM, and 0.2M-KCl was included. Citrate synthase activity is affected by ionic strength and the presence of KCl served to nullify small changes in ionic strength produced by the addition of nucleotides. A consequence of the inclusion of KCl was that slightly greater inhibitor concentrations were necessary. Care was taken to ensure that solutions of all reaction components were adjusted to pH8.0.

Results and discussion

Three different citrate synthases were investigated. The pig heart enzyme is typical of that from eukaryotic sources, the *B. megaterium* enzyme is a representative Gram-positive bacterial citrate synthase and the *Ps. aeruginosa* enzyme is typical of Gram-negative species. Multiple inhibition was studied by using the three inhibitors bromoacetyl-CoA, ATP and NADH, all of which are strictly competitive with acetyl-CoA for each of the enzymes examined. Table 1 lists the K_i values determined for each enzyme–inhibitor combination. Bromoacetyl-CoA was used because it is a substrate, albeit a poor one, for citrate synthase, and its inhibitory action may therefore reasonably be assumed to result from competition with acetyl-CoA for the active site. The inhibitors were used in the pairs ATP+bromoacetyl-CoA and ATP+NADH. In each case the concentration of one inhibitor was varied at different fixed concentrations of the other, and the reciprocal of the measured rate was plotted against the concentration of the variable inhibitor.

Table 1. *Competitive-inhibition constants for citrate synthases*

Assay conditions were the same as those described in the text for multiple-inhibition studies except that the concentration of acetyl-CoA was varied. Double-reciprocal plots were obtained at several inhibitor concentrations and K_i values derived from the replots of the slopes of these lines versus inhibitor concentration.

Enzyme source	Inhibitor	K_i (μM)
B. megaterium	Bromoacetyl-CoA	8.7
	ATP	2800
	NADH	3900
Pig heart	Bromoacetyl-CoA	25.7
	ATP	6800
	NADH	8300
Ps. aeruginosa (native enzyme)	Bromoacetyl-CoA	3.4
	ATP	2600
	NADH	80
Ps. aeruginosa (desensitized enzyme)	Bromoacetyl-CoA	3.8
	ATP	3700
	NADH	16400

As Yonetani & Theorell (1964) have shown, parallel lines in such plots indicate that the inhibitors interact with the same site of the enzyme, whereas different sites are indicated by a pattern of intersecting lines.

The results are displayed in Fig. 1. The parallel-line plots obtained in all cases with the pig heart and *B. megaterium* enzymes suggest that ATP, NADH and bromoacetyl-CoA interact with the same enzyme site. In view of the likely identity of the bromoacetyl-CoA site, it may be concluded that these results indicate that the site for both ATP and NADH is the active site on the enzyme to which the substrate acetyl-CoA normally binds. For the *Ps. aeruginosa* enzyme, the parallel-line plots again suggest that ATP and bromoacetyl-CoA have a common binding site. However, the pattern of intersecting lines obtained with this enzyme and the pair of inhibitors ATP+NADH clearly indicates a different situation from that with the other two citrate synthases. It suggests that NADH and ATP interact with different sites on the enzyme, thereby supporting our view that the NADH inhibition observed with Gram-negative bacterial citrate synthases is an allosteric, rather than an isosteric, effect.

Ps. aeruginosa citrate synthase may be desensitized to its specific NADH inhibition by treatment with 5,5'-dithiobis-(2-nitrobenzoate) (P. D. J. Weitzman, unpublished work). We have pursued this finding by examining the sensitivity of this treated enzyme to general nucleotide inhibition. Treatment of *Ps. aeruginosa* citrate synthase with 0.1mM-5,5'-dithiobis-(2-nitrobenzoate) resulted in complete loss of its normal high sensitivity to inhibition by NADH. On the other hand, the inhibition of activity by ATP was

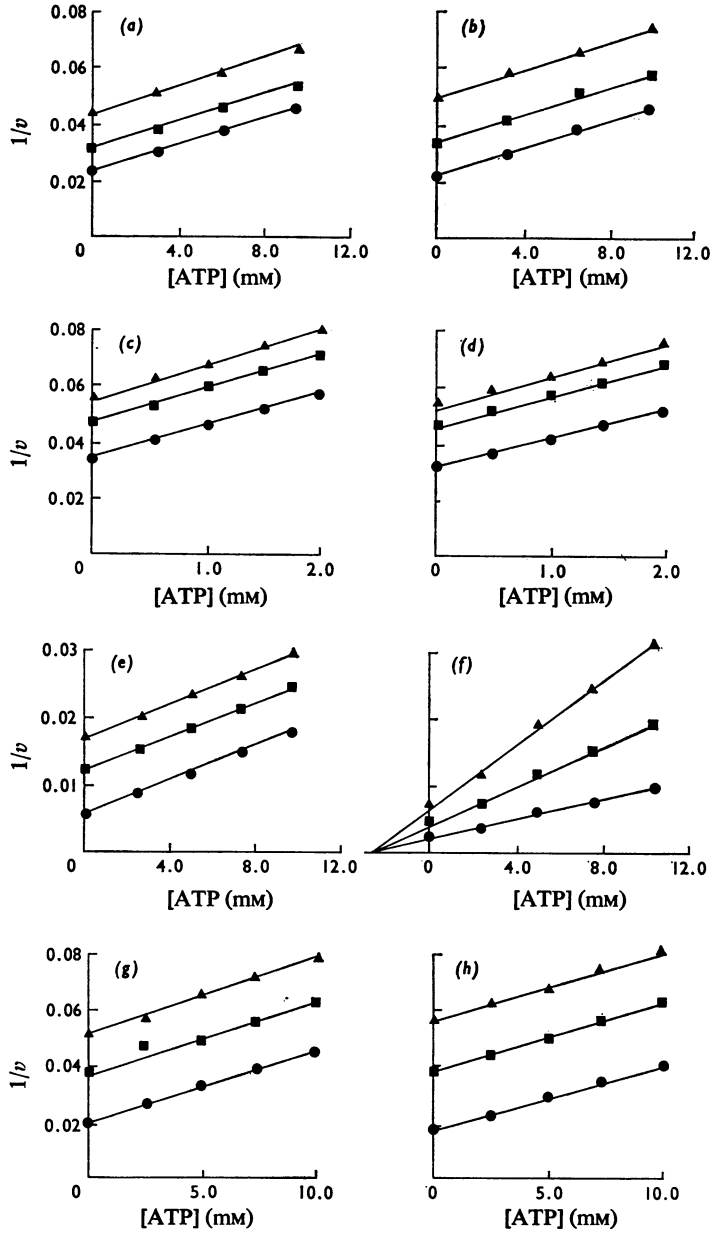

Fig. 1. *Multiple-inhibition analysis of citrate synthases*

Assay conditions are described in the text. (*a*) ATP inhibition of pig heart citrate synthase in the presence of 0(●), 15.0μM- (■) and 37.5μM- (▲) bromoacetyl-CoA. (*b*) ATP inhibition of pig heart citrate synthase in the presence of 0 (●), 5.0mM- (■) and 10.0mM- (▲) NADH. (*c*) ATP inhibition of *B. megaterium* citrate synthase in the presence of 0 (●), 3.0μM- (■) and 6.0μM- (▲) bromoacetyl-CoA. (*d*) ATP inhibition of *B. megaterium* citrate synthase in the presence of 0 (●), 15.0 mM- (■) and 30.0mM- (▲) NADH. (*e*) ATP inhibition of native *Ps. aeruginosa* citrate synthase in the presence of 0 (●), 5.0μM- (■) and 10.0μM- (▲) bromoacetyl-CoA. (*f*) ATP inhibition of native *Ps. aeruginosa* citrate synthase in the presence of 0 (●), 0.5mM- (●) and 1.0mM- (▲) NADH. (*g*) ATP inhibition of desensitized *Ps. aeruginosa* citrate synthase in the presence of 0 (●), 5.0μM- (■) and 10.0μM- (▲) bromoacetyl-CoA. (*h*) ATP inhibition of desensitized *Ps. aeruginosa* citrate synthase in the presence of 0 (●), 10.0mM- (■) and 20.0mM- (▲) NADH.

not affected by this pretreatment. These results, too, indicate the presence of an allosteric site for NADH which is distinct from the site at which ATP interacts with the enzyme. Similar conclusions have been reached for *Escherichia coli* citrate synthase; the desensitization to NADH by KCl (Weitzman, 1966) results in an enhanced sensitivity to ATP inhibition (Srere, 1968), suggesting different sites.

When multiple-inhibition studies were performed on the desensitized *Ps. aeruginosa* enzyme, only parallel-line plots were obtained (Fig. 1). It should be noted that, although treatment of the enzyme with 5,5′-dithiobis-(2-nitrobenzoate) destroys the allosteric response to NADH, inhibition can still be demonstrated at very much higher NADH concentrations. The K_I value for the desensitized enzyme is increased 200-fold over that for the native enzyme (see Table 1). The results indicate that this decreased sensitivity to NADH is similar to that exhibited by the pig heart and *B. megaterium* enzymes and, like these, is exerted isosterically.

In conclusion, the results presented here support the view that the nucleotide inhibition of eukaryotic and Gram-positive bacterial citrate synthases is an isosteric effect, in contrast with the superficially similar NADH inhibition of Gram-negative bacterial citrate synthases, which is an allosteric effect. The distinctive property of these latter enzymes in having a specific regulatory site for NADH is therefore emphasized.

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