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Feedback Inhibition of an Allosteric Triphosphopyridine Nucleotide-specific Isocitrate Dehydrogenase*

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SUMMARY

Evidence is presented for the concerted inhibition of a TPN+-specific isocitrate dehydrogenase from *Crithidia fasciculata* by two biosynthetic intermediates, oxalacetate and glyoxylate. This inhibition is competitive with substrate, and the presence of either inhibitory compound greatly increases the affinity of the enzyme for the second. This inhibition has been shown not to be due to the formation of oxalomalate, the nonenzymatic condensation product. Structural analogues of the substrate are not inhibitory, so that steric configurational analogy is apparently not the mechanism of inhibition. In view of this, it is believed that an alteration in the protein must be induced by the combination of the two inhibitors, as has been demonstrated previously with this enzyme for ATP. This work confirms the allosteric nature of the ATP inhibition and substantiates the requirement for 2 moles of ATP per mole of enzyme.

Since isocitrate can either enter the glyoxylate cycle or be metabolized via the tricarboxylic acid cycle, it is believed that the inhibition of this enzyme by oxalacetate and glyoxylate has biological significance.

Earlier studies (1) with the TPN+-specific isocitrate dehydrogenase from the protozoan *Crithidia fasciculata* demonstrated that it was subject to inhibition by nucleoside triphosphates. A more detailed investigation of this inhibition showed that it was cumulative in nature and appeared to require 2 moles of ATP per mole of enzyme. Kinetic studies indicated that the inhibitor was acting at a site other than the substrate site and that chelation was not the mechanism of inhibition.

Since these studies were, to our knowledge, the first demonstration that a TPN+-specific isocitrate dehydrogenase was subject to allosteric inhibition by nucleotides, we felt it desirable to investigate this system further. Shiio and Ozaki (2) recently demonstrated that several TPN+-isocitrate dehydrogenases were inhibited by oxalacetate and glyoxylate, competitive with isocitrate. We therefore examined the effect of these compounds on this enzyme from *Crithidia* and showed that they were inhibitory and competitive with the substrate (3). In this report the two types of inhibitors are compared to show that simultaneous inhibition by nucleotides and α-keto acids can occur. This substantiates the previous data (1) which indicated that the nucleotides were acting at a locus other than that occupied by the substrate and confirms the requirement for 2 moles of ATP to inhibit the enzyme. The concerted inhibition produced by oxalacetate and glyoxylate is not competitive with that produced by ATP, and the former do not alter the affinity of the enzyme for the latter. The possibility that this may be an artificial inhibition due to the "creation" of an alternate substrate from two smaller molecules, as described by Inagami (4) and Inagami and Mitsuda (5), has been examined and is considered not to be the case. We believe, therefore, that this cumulative nucleotide inhibition and concerted inhibition by biosynthetic intermediates has biological significance. This is discussed in relation to the position of the isocitrate dehydrogenase in cell metabolism and the possibilities for alteration of lipid and carbohydrate metabolism by inhibition of the cytoplasmic TPN+-specific isocitrate dehydrogenase.

EXPERIMENTAL PROCEDURE

Materials—All chemicals except isocitrate were purchased from Sigma. Isocitrate was prepared either from the lactone purchased from Calbiochem (1), or from the trisodium salt (Sigma). As used in this report, isocitrate refers to the *threo*-D_sL_s-racemic mixture (6) unless otherwise specified. The actual concentration of the active *threo*-D_s isomer in each solution was determined enzymatically and found to be 50% of the total isocitrate concentration in all instances.

Enzyme Preparation—Enzyme was isolated from *C. fasciculata* as previously described (1).

Enzyme Assay—The reduction of TPN+ was measured at 340 mμ in a Gilford recording spectrophotometer equipped with a Beckman DU monochromator with full scale deflections of 0 to 0.1 optical density unit. Reactions were carried out at 25° and were started by the addition of enzyme.

One unit of activity is defined as the reduction of 0.1 μmole of TPN+ per mg of protein per min at 25°.

Protein was determined by the method of Lowry *et al.* (7) or Warburg and Christian (8).

RESULTS

Concerted Inhibition by Oxalacetate and Glyoxylate—As shown in Table I, the presence of both oxalacetate and glyoxylate is required for inhibition of the reaction. Oxalacetate alone shows

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TABLE I

Concerted inhibition by oxalacetate and glyoxylate

Reaction mixtures contained 0.5 mmole of Tris-HCl, pH 7.7; 0.2 μmole of $MnCl_2$; 0.3 μmole of $NADP^+$; 0.2 μmole of isocitrate; and water to a final volume of 3 ml. Reactions were carried out with either oxalacetate or glyoxylate in a final concentration of 1 mM. Reactions were started by the addition of 22.6 μg of extract protein.

Addition	$\Delta A_{340} \times$ $min^{-1} \times 10^3$	Inhibition
		%
None	42	
Oxalacetate	39	8
Glyoxylate	42	0
Oxalacetate and glyoxylate	0	100

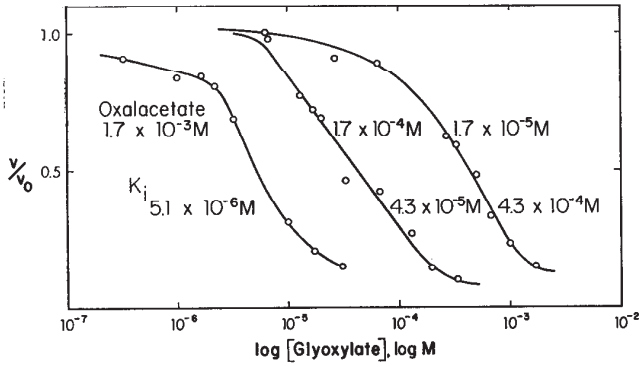

FIG. 1. Effect of oxalacetate on glyoxylate inhibition. Reaction mixtures contained 0.5 mmole of Tris-HCl buffer, pH 7.7, 0.2 μmole of $MnCl_2$, 0.3 μmole of TPN^+, 0.2 μmole of DL-isocitrate; oxalacetate and glyoxylate as indicated, and H_2O to 3 ml. Reactions were started by the addition of 45 μg of protein. V_0 was taken as the rate in the presence of oxalacetate alone, and corresponded to an inhibition of 0, 0, and 31%, respectively, for the concentrations 1.7×10^{-5} M, 1.7×10^{-4} M, and 1.7×10^{-3} M.

competitive inhibition with respect to isocitrate and has an apparent K_i of 2.7 mM. Glyoxylate alone produces some slight inhibition at a concentration of 5 mM. Neither compound will inhibit at concentrations which are physiological. When oxalacetate and glyoxylate are present together, there is a striking decrease in the K_i to 36 μM. This concerted inhibition has been described in detail in a previous report (3).

Effect of Oxalomalate on Reaction—Since oxalacetate and glyoxylate will, in the presence of cations and mildly alkaline conditions, form a nonenzymatic condensation product, experiments were carried out to determine whether this product might be the true inhibitor. The condensation product, oxalomalate, was prepared as previously described (2) and tested. Calculations made from previously determined rates of formation of the product (2) indicated that when oxalacetate and glyoxylate are present in the reaction mixture at a concentration of 1 mM each, the maximum concentration of oxalomalate which could accumulate in 30 sec, the time period of the assay, was 3 μM. However, this amount of oxalomalate is without effect, and a concentration of 333 μM condensation product inhibits the enzyme by only 9%. The latter concentration of oxalacetate and glyoxylate gives 75% inhibition. Increasing concentrations of oxalomalate resulted in 50% inhibition of the reaction at a concentration of 3 mM. The K_i for oxalacetate and glyoxylate, however, is 36 μM. Further, no oxalomalate could be detected at 1 min in a reaction mixture in which oxalacetate and glyoxylate were present at a concentration of 1 mM each. This concentration of oxalacetate and glyoxylate gave 100% inhibition. The above results indicate clearly that inhibition of the enzyme is not due to the formation of oxalomalate in the reaction mixture. This conclusion is also supported by the observations that when either oxalacetate or glyoxylate is present in the reaction mixture, the addition of the other compound results in an immediate inhibition which remains constant. The lack of time dependence and the constancy of the degree of inhibition indicate that the formation of another compound prior to inhibition of the enzyme is unlikely.

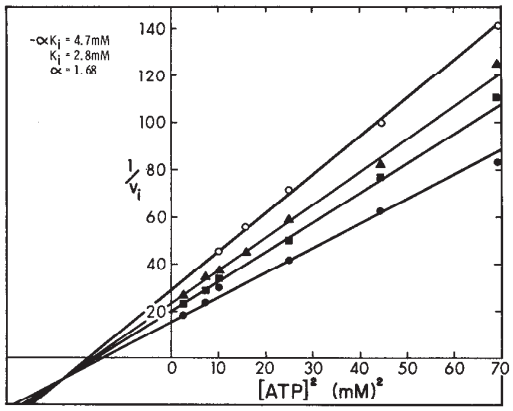

FIG. 2. Lack of interaction of ATP and oxalacetate + glyoxylate. Reaction mixtures contained 0.5 mmole of Tris-HCl buffer, pH 7.7, 0.2 μmole of $MnCl_2$, 0.26 μmole of TPN^+, 0.2 μmole of DL-isocitrate, ATP as indicated, and H_2O to 3 ml. Reactions were started by the addition of 45 μg of protein. Concentrations of inhibitors are as follows: ○——○: oxalacetate, 1 mM; glyoxylate, 0.01 mM; ▲——▲: oxalacetate, 0.03 mM; glyoxylate, 0.03 mM ■——■: oxalacetate, 0.01 mM; glyoxylate, 1 mM; ●——●: no oxalacetate or glyoxylate. $1/V_i$ is the reciprocal of $\Delta A_{340} \times min^{-1}$.

Effect of Oxalacetate on Glyoxylate Inhibition—Fig. 1 illustrates the augmentation of the inhibitory effect of the one compound by the other. Increasing the concentration of oxalacetate 10-fold decreases the apparent K_i (taken as the concentration required for 50% inhibition) for glyoxylate by about the same amount. At high concentrations of oxalacetate the requirement for glyoxylate reaches extremely low levels. Equimolar concentrations of the two compounds are not required for inhibition of the reaction and tend to make the construction of an artificial substrate less likely (*vide infra*). n values calculated according to the method of Taketa and Pogell (9) for all curves were about 1.5. This is in agreement with previous data which indicated that the reaction is first order in substrate (1). The fact that the n values are substantially greater than 1 is probably due to augmentation of the glyoxylate inhibition by oxalacetate and vice versa.

Lack of Interaction of ATP and Oxalacetate + Glyoxylate—Since both nucleotides and other compounds act on this enzyme, it was of interest to determine whether they interacted with one another as well. Fig. 2 shows the effect of ATP inhibition in the presence of three different concentrations of oxalacetate and glyoxylate. The graphical method for determining the various

TABLE II

Concerted inhibition of isocitrate dehydrogenase by glyoxylate and oxalacetate

Reaction mixtures contained 0.5 mmole of Tris-HCl, pH 7.7; 0.2 μmole of $MnCl_2$; 0.3 μmole of $NADP^+$; 0.2 μmole of isocitrate; and water to a final volume of 3 ml. Reactions were carried out with either oxalacetate or glyoxylate in a final concentration of 1 mM, and all other inhibitors were added at the same final concentration. Reactions were started by the addition of 38 μg of extract protein.

Addition	Glyoxylate		Oxalacetate	
	$\Delta A_{340} \times min^{-1} \times 10^3$	Inhibition	$\Delta A_{340} \times min^{-1} \times 10^3$	Inhibition
		%		%
None	62	0	58	0
Oxalacetate	2	97	50	14
Glyoxylate	62	0	2	97
α-Ketoglutarate	53	15	50	14
L$_s$-Malate	52	16	52	11
D$_s$-Malate	62	0	51	12
Succinate	54	13	58	0
Fumarate	57	8	56	3
Pyruvate	62	0	52	11
Acetate	62	0	56	3
Acetaldehyde	60	3	55	5
Glycolic acid	57	8	58	0
Glycolaldehyde	58	6	58	0
Oxalate	62	0	53	9
Ethanol	62	0	53	9
Formate	62	0	53	9
Formaldehyde	62	0	58	0

constants is essentially a variation of that described by Dixon (10). The proof for this method has been discussed in theory by Webb (11) and by Yonetani and Theorell (12) with respect to the liver alcohol dehydrogenase. The slope of the lines varies inversely with α, an interaction constant which is a measure of the interaction of the inhibitors I_1 and I_2 in the EI_1I_2 complex. In a plot of $1/V_i$ against I_1 with I_2 as the fixed changing variable, a series of straight lines will result which intersect at $-\alpha K_{EI_1}$ on the abscissa. When the inhibition is purely competitive $\alpha = \infty$ and the slope remains constant since

$$\text{Slope} = \left[1 + \frac{I_2}{\alpha K_{EI_2}}\right] \frac{K_m}{SV_m K_{EI_1}}$$

It may also be shown that for $\infty > \alpha > 0$ and $\alpha \neq 1$, positive ($\alpha < 1$) or negative ($\alpha > 1$) interactions exist between I_1 and I_2 in the EI_1I_2 complex (11). When $\alpha = 1$, there is no interaction between I_1 and I_2.

In Fig. 2, $-\alpha K_{EI_1} = -4.7$ mM. K_{EI_1} for ATP was determined separately in the absence of oxalacetate and glyoxylate and was shown to be 2.8 mM. α is then equal to 1.68, indicating that there is no competitive interaction between the nucleotides and the α-keto acids, as would be expected if they acted at the same site.

This confirms previous data (1), which indicated that ATP was a noncompetitive inhibitor of the substrate, and is in agreement with the other studies (3), which showed that oxalacetate and glyoxylate were competitive inhibitors of the substrate. The fact that $1/V_i$ must be plotted against $[ATP]^2$ in order to obtain straight lines confirms our earlier data (1) in which n values close to 2 were obtained for plots of log $[v/V_0 - v]$ against log $[I]$. Two moles of ATP appear to be required for inhibition of the enzyme, whereas all other reactants are first order.

TABLE III

Effect of structural analogues of threo-D$_s$-isocitrate

Reaction mixtures contained 0.5 mmole of Tris-HCl, pH 7.7; 0.2 μmole of $MnCl_2$; 0.3 μmole of $NADP^+$; 0.2 μmole of isocitrate; and water to a final volume of 3 ml. All inhibitors were present in a final concentration of 1 mM. Reactions were started by the addition of 45.2 μg of protein.

Addition	$\Delta A_{340} \times min^{-1} \times 10^3$
None	80
D$_s$-Malate + formate	83
D$_s$-Malate + formaldehyde	76
D$_s$-Malate + CO_2	78
α-Ketoglutarate	64
α-Ketoglutarate + formate	64
α-Ketoglutarate + formaldehyde	62
α-Ketoglutarate + CO_2	61
Oxalacetate	72
Oxalacetate + CO_2	70

Effect of Structural Analogues of Oxalacetate and Glyoxylate on Isocitrate Dehydrogenase—In view of the evidence brought forward by Koshland (13) for the "induced fit" theory of enzyme substrate interactions, we believed that the concerted inhibition by oxalacetate and glyoxylate might be a result of the construction of an artificial substrate which had a steric configuration similar to *threo*-D$_s$-isocitrate (6). The use of analogues to form an artificial substrate has been demonstrated by Inagami (4) and Inagami and Mitsuda (5) with trypsin. Therefore a number of other compounds were tested in an attempt to construct a substrate from two smaller molecules. Table II lists these compounds. They were added to the reaction mixture in the presence of oxalacetate or glyoxylate, as indicated. Although not indicated in the table, addition of these compounds in series did not result in inhibition unless both oxalacetate and glyoxylate were present.

Because of the apparent specificity of the latter two compounds, the steric configurations of *threo*-D$_s$-isocitrate, oxalacetate, and glyoxylate were examined by the use of Corey-Pauling Koltun models. Although some structural similarities were evident, there was not a close correspondence between *threo*-D$_s$-isocitrate and the combination of oxalacetate and glyoxylate. Models of other combinations, D$_s$-malate and formate, formaldehyde, or CO_2, resembled *threo*-D$_s$-isocitrate much more closely, but none of these was inhibitory (Table III). Even the combination of α-ketoglutarate and CO_2, the end products of the reaction, resulted in only a 20% inhibition at a concentration of 1 mM each. This is no greater than that found for α-ketoglutarate alone and much less than the complete inhibition produced by oxalacetate and glyoxylate (Tables II and III). The presence or absence of a spatial relationship as demonstrated by models does not necessarily apply to the actual molecules. However, the inability of oxalacetate and glyoxylate to conform to the configuration of *threo*-D$_s$-isocitrate would appear to lend support to the concept that simple structural analogy is not a mechanism of inhibition for these two compounds.

DISCUSSION

Previous studies of this enzyme (1) demonstrated that it was subject to inhibition by nucleoside triphosphates. The inhibition was not at any of the substrate loci, and logarithmic plots of the inhibition curves indicated that 2 molecules of inhibitor were required. Calculations from thermodynamic data showed a substantial free energy and entropy change during the enzyme-ATP interaction without nucleotide hydrolysis. These findings led us to postulate that the ATP was acting as an allosteric inhibitor.

This communication describes the concerted inhibition of this enzyme by two biosynthetic intermediates of the tricarboxylic acid and glyoxylate cycle. The inhibition appears to be specific for these two compounds (Table II) and is not due to the synthesis of a nonenzymatic condensation product or the fortuitous construction of a sterically similar artificial substrate. The best evidence against the latter possibility is the failure to achieve comparable inhibition by α-ketoglutarate and CO_2 at concentrations considerably higher than those required for complete inhibition by the two inhibitors (Fig. 1) (Table III). The failure to inhibit with D$_s$-malate and formate (Table III), which together closely mimic *threo*-D$_s$-isocitrate, also argues against steric analogy to the substrate as a mechanism of inhibition.

However, the above data leave open the question of the mechanism of this concerted inhibition. The facts that either compound can greatly increase the affinity of the enzyme for the other (Fig. 1) and that both are required for competitive inhibition indicate that the presence of the two inhibitors makes the substrate locus inaccessible to isocitrate. This could be a result of alteration in the protein, a possibility already demonstrated for ATP (1). If this is the case, glyoxylate would be kinetically undetectable, binding by itself but not altering the rate of the reaction unless oxalacetate is also present (Table I). Preliminary binding studies, with Sephadex G-25, have shown that glyoxylate alone appears to bind the protein without alteration of the reaction. The addition of small amounts of oxalacetate to this presumed enzyme-glyoxylate complex results in a significant inhibition of 45%, whereas the equivalent amount of oxalacetate added to untreated enzyme gives only about 10 to 15% inhibition. This is also supported by the finding that oxalacetate alone will inhibit slightly but that glyoxylate alone is without effect until concentrations in excess of 5 mM are attained. These concentrations have probably no physiological significance.

Competition experiments (Fig. 2) between oxalacetate and glyoxylate and ATP have confirmed the nonidentity of the loci for the nucleotides and α-keto acids and have corroborated the requirement for 2 moles of ATP per mole of enzyme. Since the reaction is first order with respect to all other ligands, this would appear to substantiate the allosteric nature of the nucleotide inhibition.

The fact that both inhibitory molecules are intermediates of the glyoxylate cycle, which uses isocitrate as the initiating compound, raises the intriguing question of whether this represents a form of feedback inhibition of a tricarboxylic acid cycle enzyme. The aconitase equilibrium greatly favors the accumulation of citrate, and so the conversion of isocitrate to α-ketoglutarate represents the first committed step in oxidative carbohydrate metabolism. It is also the first point at which reduced pyridine nucleotides are produced, and this may provide a rationale for the inhibition by ATP. Isocitrate has two alternatives: it may go forward to α-ketoglutarate or be diverted to the glyoxylate cycle via the isocitrate lyase. In this event, the concerted inhibition by oxalacetate and glyoxylate, intermediates of the latter pathway, may represent a form of feedback inhibition of a cytoplasmic isocitrate dehydrogenase.

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