Biochimica et Biophysica Acta, 567 (1979) 453—463

BBA 68711

CARBAMYL PHOSPHATE-DEPENDENT ATP SYNTHESIS CATALYZED BY FORMYLTETRAHYDROFOLATE SYNTHETASE

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(Received September 25th, 1978)

Key words: Carbamyl phosphate; ATP synthesis; Formyltetrahydrofolate synthetase

Summary

Formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3) from *Clostridium cylindrosporum* catalyzes phosphate transfer from carbamyl phosphate to ADP. This activity is lost when monovalent cations are removed and is recovered when K^+ is added back. Carbamyl phosphate is an inhibitor of the formyltetrahydrofolate synthetase forward reaction, and formate as well as phosphate inhibit the ATP synthesis reaction. Acetyl phosphate and phosphonoacetate are inhibitors of both reactions. The results of kinetic studies support the concept that carbamyl phosphate is an analog of the putative intermediate of the formyltetrahydrofolate synthetase reaction, formyl phosphate.

Introduction

A number of mechanisms have been proposed for the reaction catalyzed by formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3), but only a concerted mechanism is supported by experimental data. There is no evidence to support the existence of freely dissociable intermediates, and the evidence for tightly bound intermediates is suggestive only [1]. In a recent report [2] we presented data which showed that formyltetrahydrofolate synthetase catalyzes the formation of ATP from carbamyl phosphate and ADP. This reaction was interpreted to be supportive of enzyme-bound formyl phosphate as an intermediate in the natural reaction catalyzed by the enzyme (Eqn. 1).

$$\text{ATP} + \text{tetrahydrofolate} + \text{HCO}^- \overset{\text{O}}{\parallel} \xrightleftharpoons{Mg^{2+},K^+} \text{10-formyltetrahydrofolate} + \text{ADP} + P_i \qquad (1)$$

The interpretation rests on the assumption that carbamyl phosphate is an analog of formyl phosphate and would substitute for the putative intermediate according to Eqns. 2 and 3.

$$\text{ATP} + \text{HC}(=\text{O})\text{O}^- \rightleftharpoons \text{ADP} + \text{HC}(=\text{O})\text{OPO}_3^{2-} \quad (2)$$

$$\text{ATP} + \text{H}_2\text{NC}(=\text{O})\text{O}^- \rightleftharpoons \text{ADP} + \text{H}_2\text{NC}(=\text{O})\text{OPO}_3^{2-} \quad (3)$$

To gain a better understanding of the carbamyl phosphate-dependent ATP formation reaction, a kinetic study of this reaction as well as related inhibition studies of the formylation of tetrahydrofolate were undertaken.

Materials and Methods

Formyltetrahydrofolate synthetase was purified from *Clostridium cylindrosporum* as described by Rabinowitz and Pricer [3] and was then chromatographed on DEAE-cellulose [4]. The crystalline enzyme migrates as a single band in sodium dodecylsulfate-polyacrylamide gel electrophoresis. Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid in aqueous solution and was purified by chromatography on DEAE-cellulose [5]. Tris base, folic acid, ADP, ATP, $NADP^+$, carbamyl phosphate, yeast hexokinase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. Phosphonoacetic acid was prepared by acid hydrolysis of triethylphosphonoacetate [6] purchased from Aldrich. All other chemicals were reagent grade. ADP and ATP concentrations were determined spectrophotometrically at 259 nm. Formate and tetrahydrofolate concentrations were determined by assay with formyltetrahydrofolate synthetase [7].

Enzyme Assays. Two different assays were used for following ATP synthesis from carbamyl phosphate and ADP. The first was a coupled assay [8]: the reaction was performed in a 1 ml volume, which included 100 mM triethanolammonium chloride (pH 8.0), 5 mM $MgCl_2$, 1 mM glucose, 50 mM KCl, 0.25 mM $NADP^+$, 100 mM 2-mercaptoethanol, 40 mM Tris-HCl, 2.5 units glucose-6-phosphate dehydrogenase, 5 units hexokinase, and 40 μg formyltetrahydrofolate synthetase. The concentrations of ADP, carbamyl phosphate and tetrahydrofolate were varied for each experiment. The reaction was measured at 340 nm in a Gilford Model 240 spectrophotometer at 37° C. In some experiments a second, direct assay was used. Carbamyl phosphate, ADP, and tetrahydrofolate were incubated at the appropriate concentrations together with 6.25 mM $MgCl_2$, 125 mM KCl, 125 mM triethanolammonium chloride (pH 8.0), 125 mM 2-mercaptoethanol and 10 μg formyltetrahydrofolate synthetase in 0.2 ml. After 5 min at 37° C, 50 μl 0.2 M sodium formate was added and the reaction was continued for another 5 min. 0.5 ml 0.36 N HCl was then added to stop the reaction and convert the formyltetrahydrofolate to 5,10-methenyltetrahydrofolate. The amount of product was determined spectrophotometrically at 350 nm after 10 min using an ϵ 24 800 $M^{-1} \cdot cm^{-1}$. In this

assay, the ATP formed in the first 5 min of incubation was quantitatively converted to ADP and P_i during the subsequent 5 min incubation. The amount of 5,10-methenyltetrahydrofolate was thus equivalent to the amount of ATP formed, after correction for a control. This control, which consisted of a 5 min incubation with formate added at zero time, measured the ATP contaminant initially present in the ADP as well as that formed in the second 5 min incubation. ATP production using this assay was linear for a 20 min period in the presence of saturating concentrations of substrates. The kinetic constants determined by the two assays were similar. Prior to each experiment, formyltetrahydrofolate synthetase was prepared by centrifuging a sample of the enzyme/50% ammonium sulfate suspension, dissolving the enzyme in 0.1 M Tris-HCl (pH 7.5), 0.1 M KCl, 0.05 M 2-mercaptoethanol, and dialyzing the sample against the buffer for 3 h to remove residual $(NH_4)_2SO_4$.

The assay for the normal reaction was done in either 0.25 ml or 1.0 ml which contained 100 mM triethanolammonium chloride (pH 8.0), 5 mM $MgCl_2$, 100 mM KCl, 200 mM 2-mercaptoethanol, 40 mM Tris-HCl, and 5—20 ng protein. ATP, tetrahydrofolate and formate were added as noted. The reaction was stopped after 10 min at 37° C by the addition of HCl to a final concentration of 0.25 N. The amount of formyltetrahydrofolate formed was measured at 350 nm.

Analysis of kinetic data. A preliminary Lineweaver-Burk plot ($1/v$ vs. $1/A$) was made for each set of data. All data which appeared linear were then processed by the weighted least-squares computer program developed by Cleland [9]. This program was adapted for use with the HP-9830 calculator and 9862A Calculator Plotter by translation from Fortran into Basic and insertion of program steps to allow direct plotting of the best-fit double-reciprocal plot. The program is designed to fit directly to a hyperbola so initial velocities and concentrations of variable substrates were input directly. Since the same enzyme concentration was used for each data point within a given set of data, equal variances were assigned to all initial velocities. The weighting factor for each velocity was therefore v_i^4 [9]. From the statistical analysis of the data, slopes (K/V), intercepts ($1/V$), K and V were obtained, all with standard errors of estimation.

Results

Effect of removing monovalent cations on the carbamyl phosphate-dependent ATP synthesis reaction

Since the rate of ATP formation from carbamyl phosphate and ADP is much slower than from formyltetrahydrofolate, P_i and ADP [2], it is important to demonstrate that a contaminating enzyme is not responsible for the reaction. The requirement for tetrahydrofolate [2], presumably playing a structural role, suggests that it is not. A more definitive experiment was done, however. Formyltetrahydrofolate synthetase dissociates into inactive monomers when specific monovalent cations are removed and reassociates to form the active tetramer when the cations are added again [1]. In Fig. 1 data are presented which shows that both enzymic activities decrease when K^+ is removed and return when K^+ is added to cause reassociation. It is interesting that the

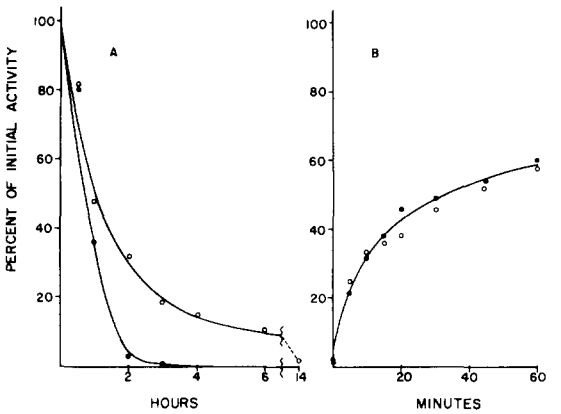

Fig. 1. Inactivation and recovery of enzymic activities for ATP (○———○) and formyltetrahydrofolate (●———●) formation. (A) Loss in activities. The protein (3 mg) was centrifuged, dissolved in 0.3 ml of 25 mM Tris-HCl, 50 mM 2-mercaptoethanol (pH 8.0) and dialyzed against the same buffer at 0°C. Samples were removed for the synthetase assay and the ATP synthesis assay using the coupled system. (B) Recovery of activities. At the end of 14 h, the dialyzed protein was diluted 1 : 10 (v/v) in buffer containing 0.2 M KCl and incubated at 20°C. Samples were removed for assay.

synthetase decreases faster and that the ATP synthesis activity persists longer. This may mean that the monomer retains some of the latter activity for a period of time.

Kinetic constants of the substrates in the carbamyl phosphate-dependent ATP formation reaction

Reciprocal plots of $1/v$ vs. $1/A$ using either carbamyl phosphate or ADP as the variable substrate were linear. Because tetrahydrofolate is an apparent activator of the reaction, its K_m as well as its effect on the K_m values of the two substrates were also determined. Plots of the primary data are presented in Fig. 2. The intersecting patterns rule out a Ping-Pong type mechanism. Increasing the concentration of tetrahydrofolate causes a significant decrease in the K_m values for carbamyl phosphate (7-fold) and ADP (10-fold). Likewise increasing the concentration of these two substrates caused the K_m of tetrahydrofolate to decrease 4-fold. Of particular interest is the reciprocal plot of the data in which carbamyl phosphate was varied at several tetrahydrofolate concentrations (Fig. 2A). Tetrahydrofolate appears to be a 'competitive activator' which means that at an infinite carbamyl phosphate concentration there is no activation by tetrahydrofolate. ADP has no effect on the K_m of carbamyl phosphate nor does the latter substrate affect the K_m of ADP. The kinetic constants of the ATP synthesis reaction are compared to those of the forward reaction and to known dissociation constants in Table 1.

Inhibitors of formyltetrahydrofolate synthetase and carbamyl phosphate-dependent ATP formation activities

If both enzymic activities are catalyzed by the same site, it would be expected that (a) carbamyl phosphate should be an inhibitor of the formation

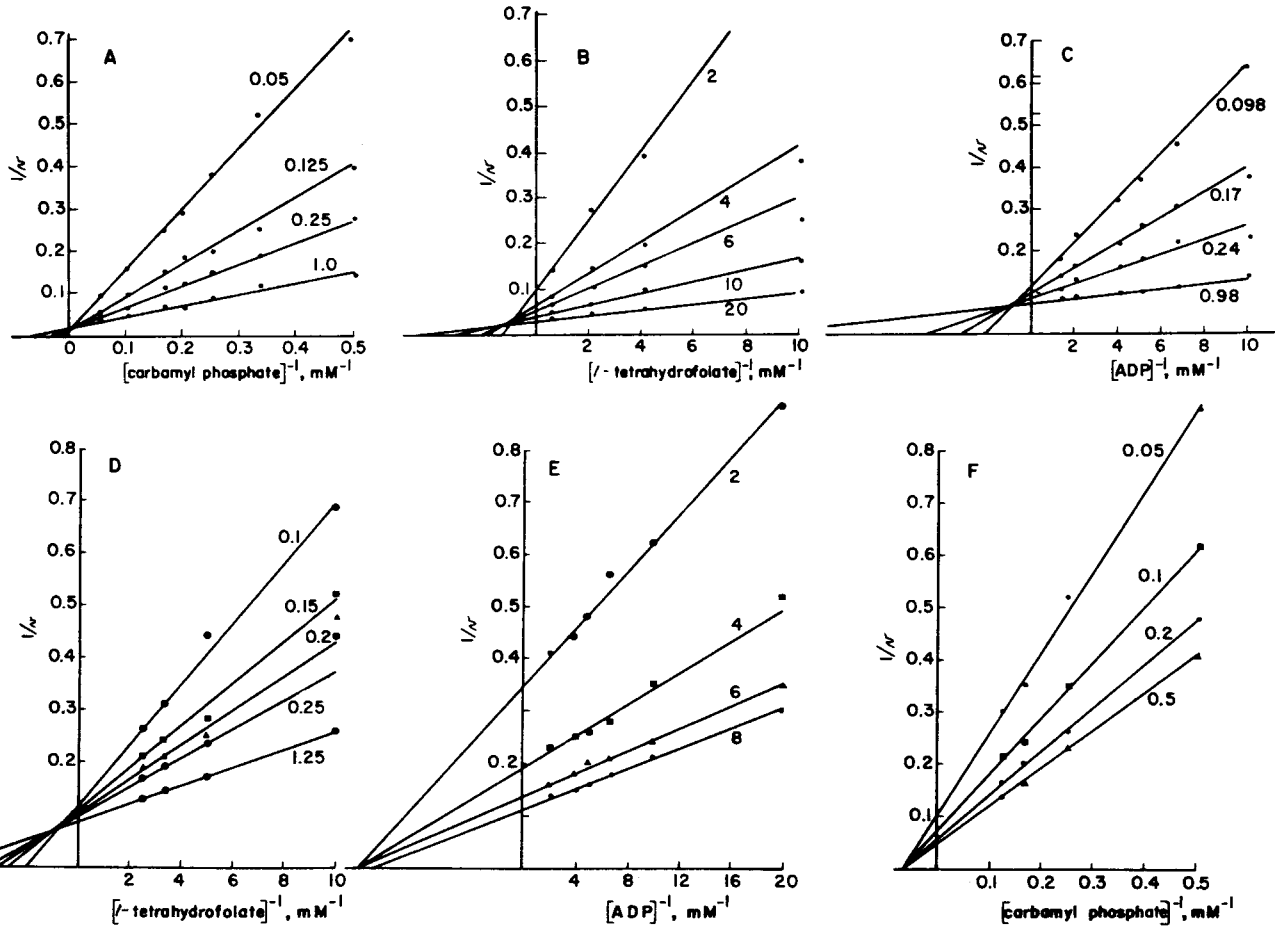

Fig. 2. Reciprocal plots of kinetic data for the ATP synthesis reaction. Assays were done using the coupled assay. Units of $1/v$ are expressed as $(\mu M/min)^{-1}$. (A) Varying carbamyl phosphate at serveral concentrations of L-tetrahydrofolate in the presence of 2 mM ADP. (B) Varying (—)-tetrahydrofolate at several concentrations of carbamyl phosphate in the presence of 2 mM ADP. (C) Varying ADP at several concentrations of (—)-tetrahydrofolate in the presence of 40 mM carbamyl phosphate. (D) Varying (—)-tetrahydrofolate at several ADP concentrations in the presence of 40 mM carbamyl phosphate. (E) Varying ADP at several carbamyl phosphate concentrations in the presence of 1 mM (—)-tetrahydrofolate. (F) Varying carbamyl phosphate at several ADP concentrations in the presence of 1 mM (—)-tetrahydrofolate.

TABLE I

COMPARISON OF K_m AND K_d VALUES OF SUBSTRATES IN THE TWO ENZYMATIC ACTIVITIES

Substrate	K_m (mM)		K_d (mM)
	ATP synthesis	Formyltetrahydrofolate synthesis	
ADP	0.032 [a]	0.13 [c]	0.089 [d]
(—)-Tetrahydrofolate	0.032—0.077 [b]	0.27	0.32 [e], 0.053 [f]
Carbamyl phosphate	11.9 [a]		

[a] Value obtained after extrapolation to infinite tetrahydrofolate concentration.
[b] Range of values from three experiments. Value obtained after extrapolation to infinite carbamyl phosphate concentration.
[c] Taken from Ref. 10.
[d] Taken from Ref. 4.
[e] Dissociation from the free enzyme. Taken from Ref. 10.
[f] Dissociation from the E-MnADP complex. Taken from Ref. 11.

of 10-formyltetrahydrofolate, (b) formate should inhibit the ATP formation reaction, and (c) inhibitory analogs of carbamyl phosphate should inhibit both activities. Such is the case. Carbamyl phosphate inhibits the formation of formyltetrahydrofolate effectively. The type of inhibition is non-competitive against each substrate (Fig. 3). The K_i value depends on the concentration of the other two substrates. For example with ATP as the variable substrate the K_i value increases from 35 to 55 mM when the formate concentration is increased from its K_m value to a saturating concentration. Increasing the tetrahydrofolate concentration from its K_m value to a saturating concentration decreases the K_i value by one-third. These results are consistent with the finding that tetrahydrofolate decreases the K_m of carbamyl phosphate in the ATP synthesis reaction and that formate is an inhibitor of the reaction (see below). When ATP and formate are varied together in a constant ratio, the inhibition is relieved at infinite concentrations of the two substrates (Fig. 3) suggesting that carbamyl phosphate occupies both the formate and ATP binding sites.

Formate is a weak inhibitor of the ATP synthesis reaction having a K_i value of about 60 mM. Acetyl phosphate and phosphonoacetate, which presumably are analogs of carbamyl phosphate and formyl phosphate, are inhibitors of both reactions. Acetyl phosphate is a competitive inhibitor of carbamyl phosphate in the ATP synthesis reaction (Fig. 4A) with a K_i of 7 mM and an inhibitor in the 10-formyltetrahydrofolate synthetase reaction. The latter inhibition can be relieved when formate and ATP are present in saturating concentrations (Fig. 4B). Acetyl phosphate did not function as a substrate in the ATP formation reaction under the conditions used. The fact that carbamyl phosphate is a fair substrate whereas acetyl phosphate is not may be explained on the basis of energetics. The product of the reaction with carbamyl phosphate, carbamate, would be easily hydrolyzed thus displacing the equilibrium of the reaction. In the case of acetyl phosphate this addition driving force would not be present.

Phosphonoacetate inhibition of the forward reaction requires fairly high concentrations but can also be relieved by saturating concentrations of formate

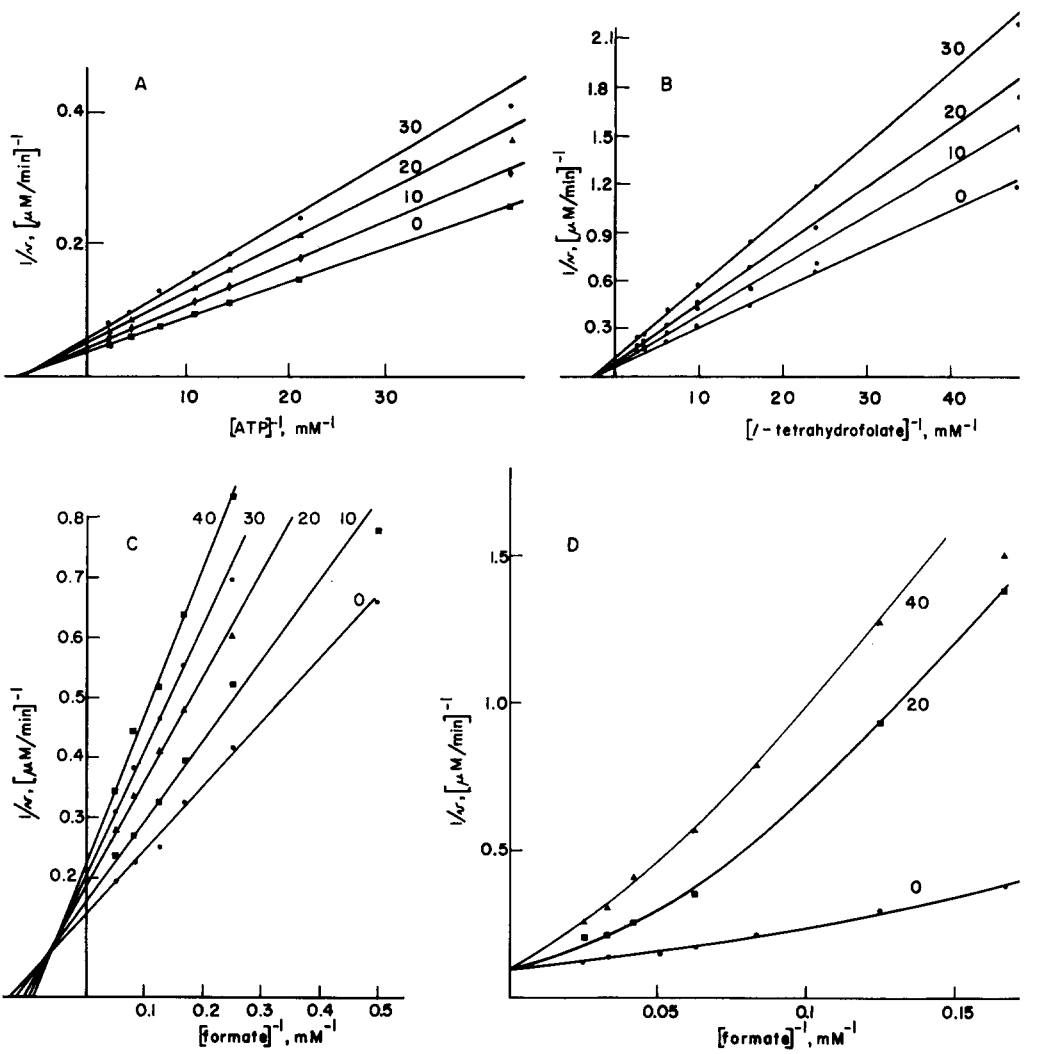

Fig. 3. Inhibition of the formyltetrahydrofolate synthetase reaction by carbamyl phosphate. (A) Varying ATP at 1 mM (—)-tetrahydrofolate and 40 mM formate. (B) Varying (—)-tetrahydrofolate at 40 mM formate and 0.24 mM ATP. (C) Varying formate at 1 mM (—)-tetrahydrofolate and 0.25 mM ATP. (D) Varying formate, $MgCl_2$ and ATP in a constant ratio of 40 : 2 : 1 at 0.5 mM (—)-tetrahydrofolate. The concentrations of carbamyl phosphate (mM) are shown.

and ATP (Fig. 5A). Acetate at the same concentrations had no inhibitory action. Phosphonoacetate is a non-competitive inhibitor of each substrate, with K_i values about 50 mM, however the inhibition is complex, giving parabolic replots of slope and intercept. In the ATP synthesis reaction, it is a non-competitive inhibitor of carbamyl phosphate (Fig. 5B) having a K_i between 20—40 mM. Perhaps it can inhibit in two ways: as a competitor of carbamyl phosphate and as an analog of phosphate.

Inorganic phosphate, which is a product inhibitor of the 10-formyltetrahydrofolate synthetase reaction also inhibits the ATP formation reaction. It is

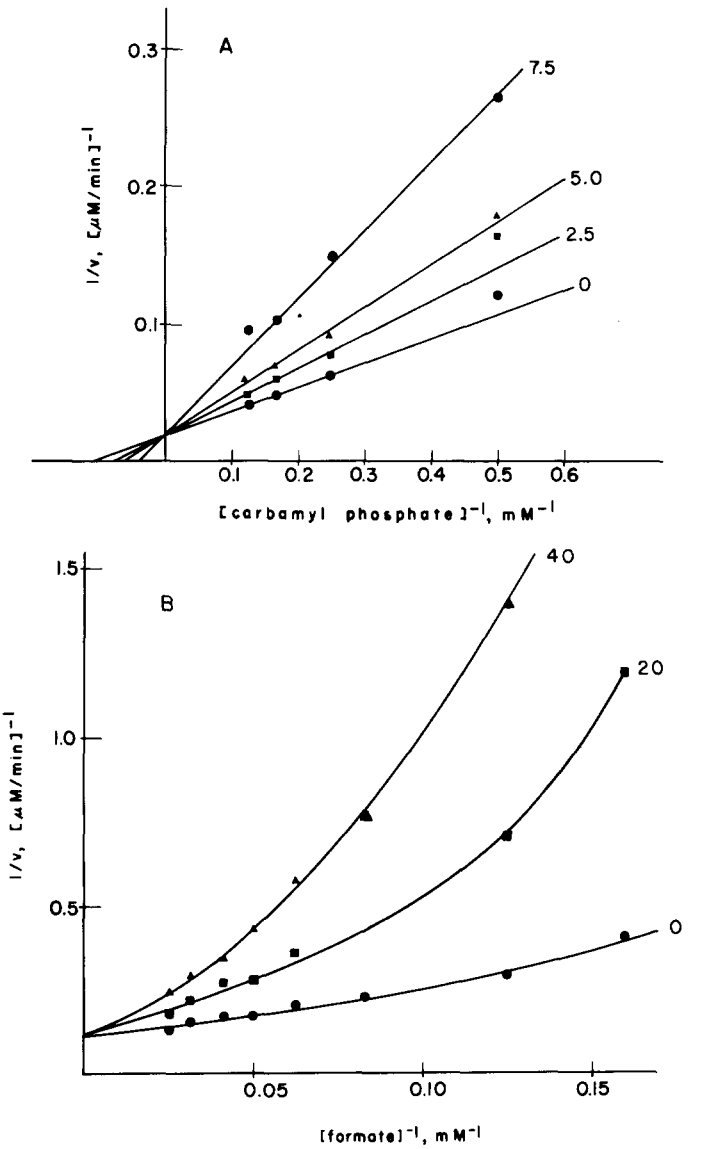

Fig. 4. Inhibition of both reactions by acetyl phosphate. (A) The ATP synthesis reaction. The reaction was done using the uncoupled, direct assay varying carbamyl phosphate in the presence of 1.25 mM ADP and at several concentrations of acetyl phosphate (mM). (B) Formyltetrahydrofolate synthetase reaction. Formate, $MgCl_2$, and ATP were varied in a constant ratio of 40 : 2 : 1 in the presence of a constant concentration of (—)-tetrahydrofolate at 0.5 mM, and the concentrations of acetyl phosphate (mM) shown.

an uncompetitive inhibitor of ADP and a non-competitive inhibitor of carbamyl phosphate with K_i values of 12 mM and 2 mM, respectively. Such a result might be expected if phosphate acts as a dead-end inhibitor and adds to an E · ADP complex before carbamyl phosphate either in an ordered or random sequence [12].

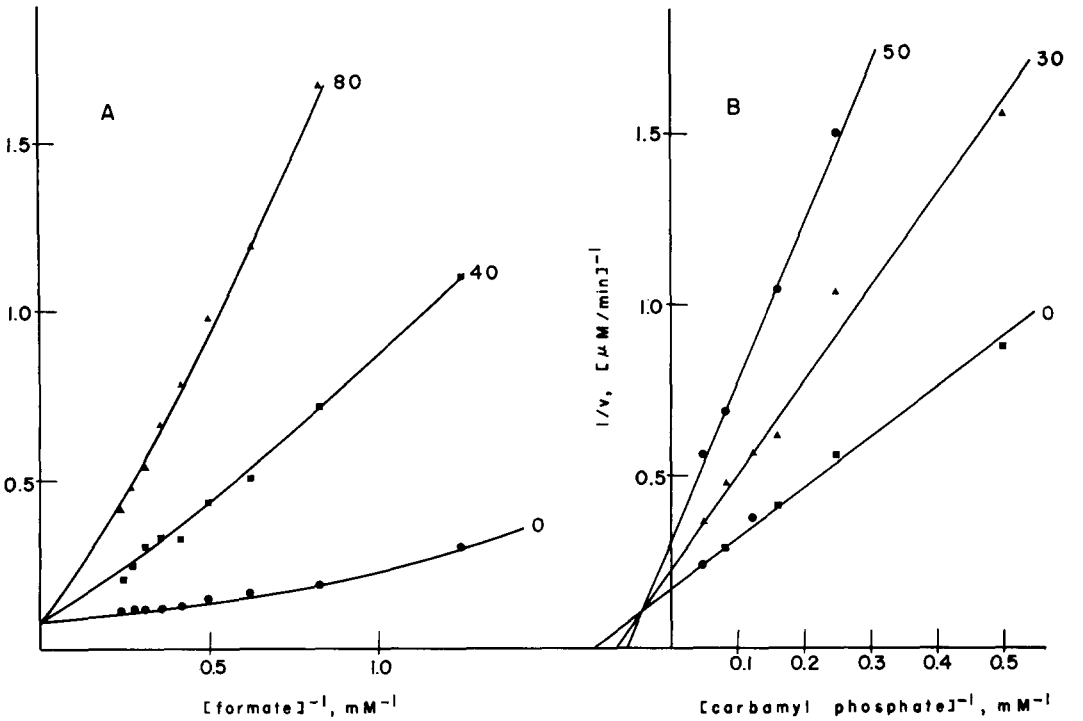

Fig. 5. Inhibition of both reactions by phosphonoacetate. (A) Formyltetrahydrofolate synthetase reaction. Formate, $MgCl_2$ and ATP were varied in a constant ratio of 40 : 2 : 1 (w/w) in the presence of 0.05 mM (—)-tetrahydrofolate and the concentrations of phosphonoacetate (mM) shown. (B) ATP synthesis. The coupled assay was used. Carbamyl phosphate was varied in the presence of 1 mM (—)-tetrahydrofolate and 1.25 mM ADP and the concentrations of phosphonoacetate (mM) shown.

Discussion

The data presented in this report clearly show that the carbamyl phosphate-dependent ATP synthesis reaction catalyzed by 10-formyltetrahydrofolate synthetase is a property of this enzyme's catalytic center. Evidence for this conclusion includes the observation that both catalytic activities are lost upon dialysis against buffer lacking a requisite monovalent cation and are subsequently recovered upon incubation with K^+. In addition, the similarity of kinetic constants for common reactants and inhibitors and the fact that substrates of one reaction are inhibitors of the other also supports this conclusion. For example, carbamyl phosphate inhibits the formation of 10-formyltetrahydrofolate and formate inhibits the synthesis of ATP. Acetyl phosphate, phosphonoacetate and phosphate inhibit both activities. The K_m values of ADP and tetrahydrofolate in the ATP synthesis reaction are in reasonable agreement with their dissociations constants. The K_m of tetrahydrofolate is in very good agreement when it is compared to the K_d of the E · MnADP-tetrahydrofolate complex, a valid comparison since ADP is present in the K_m determination.

Significantly, the inhibition of the 10-formyltetrahydrofolate synthetase

reaction by carbamyl phosphate, acetyl phosphate, and phosphonoacetate can only be eliminated when both ATP and formate are present at infinite concentrations, suggesting that these inhibitors bind to sites normally occupied by both substrates. This provides support for the idea that these compounds are analogs of formyl phosphate, a proposed intermediate in the reaction.

Tetrahydrofolate must play a structural role in the ATP synthesis reaction. There is good evidence based on NMR and EPR [11,13] studies that the binding of this substrate to the ternary enzyme-MnATP complex alters the conformation of the protein. The suggestion has been made that the active site is not catalytically competent until all the substrates have been bound [11]. Tetrahydrofolate may have a large role in the promotion of the catalytically functional active site structure. An alternative explanation of the requirement for tetrahydrofolate is that it actually takes part in the reaction. For example, it is conceivable that phosphate is transferred to this substrate from carbamyl phosphate and then to ADP. This would require the synthetase reaction to be quite complex if carbamyl phosphate is an analog of formyl phosphate; the synthesis of phosphoryl tetrahydrofolate would be followed by formation of formyl phosphate and then 10-formyltetrahydrofolate. We tested for this possibility by determining whether tetrahydrolfolate caused the hydrolysis of carbamyl phosphate in the absence of ADP but in the presence of the enzyme. We reasoned that if phosphate transfer occurred the carbamate formed would immediately hydrolyze, pulling the reaction to completion. The results were negative. Another argument in support of an activator rather than a substrate role for tetrahydrofolate in the ATP synthesis reaction comes from the data in Fig. 2A, which suggests that at an infinite concentration of carbamyl phosphate tetrahydrofolate is not required.

ATP synthesis from carbamyl phosphate and ADP has also been observed with glutamine synthetase [14] and biotin carboxylase [15]. In each case carbamyl phosphate is presumed to be an analog of a putative carboxy phosphate anhydride intermediate. Interestingly biotin carboxylase requires the presence of biotin for this activity, similar to the requirement for tetrahydrofolate by 10-formyltetrahydrofolate synthetase.

Acknowledgements

This work was supported in part from research grants to R.H.H. from the University of Kansas and NIH (AM 07140) and from a Cottrell Research Grant to D.H.B. from Research Corporation.

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