is effectively hydration of cyanate to give carbamate (Anderson, 1980). There is no kinetic evidence for positive or negative cooperativity that might be mediated through subunit interactions of an oligomeric structure (Anderson & Little, 1986). Although the biological role of cyanase is unknown, it is difficult to envisage the need for extensive quaternary structure related to a metabolic regulatory role involving an induced enzyme that catalyzes decomposition of cyanate. The oligomeric structure is very stable, and there is no evidence that the enzyme undergoes dissociation under normal conditions (Anderson, 1980). However, preliminary studies in our laboratory indicate that reversible dissociation to inactive dimers can be demonstrated by covalent modification reactions, suggesting that the oligomeric structure is required for catalytic activity.[3] The involvement of bicarbonate as a substrate in this reaction and the observation of apparent half-site binding may be related to the oligomeric structure, perhaps involving intersubunit binding sites.

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Substrate Activity of Synthetic Formyl Phosphate in the Reaction Catalyzed by Formyltetrahydrofolate Synthetase†

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Received November 19, 1986; Revised Manuscript Received February 19, 1987

ABSTRACT: Formyl phosphate, a putative enzyme-bound intermediate in the reaction catalyzed by formyltetrahydrofolate synthetase (EC 6.3.4.3), was synthesized from formyl fluoride and inorganic phosphate [Jaenicke, L. v., & Koch, J. (1963) *Justus Liebigs Ann. Chem. 663*, 50–58], and the product was characterized by ^{31}P, ^{1}H, and ^{13}C nuclear magnetic resonance (NMR). Measurement of hydrolysis rates by ^{31}P NMR indicates that formyl phosphate is particularly labile, with a half-life of 48 min in a buffered neutral solution at 20 °C. At pH 7, hydrolysis occurs with P–O bond cleavage, as demonstrated by ^{18}O incorporation from $H_2{}^{18}O$ into P_i, while at pH 1 and pH 13 hydrolysis occurs with C–O bond cleavage. The substrate activity of formyl phosphate was tested in the reaction catalyzed by formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum*. Formyl phosphate supports the reaction in both the forward and reverse directions. Thus, N^{10}-formyltetrahydrofolate is produced from tetrahydrofolate and formyl phosphate in a reaction mixture that contains enzyme, Mg(II), and ADP, and ATP is produced from formyl phosphate and ADP with enzyme, Mg(II), and tetrahydrofolate present. The requirements for ADP and for tetrahydrofolate as cofactors in these reactions are consistent with previous steady-state kinetic and isotope exchange studies, which demonstrated that all substrate subsites must be occupied prior to catalysis. The k_{cat} values for both the forward and reverse directions, with formyl phosphate as the substrate, are much lower than those for the normal forward and reverse reactions. Kinetic analysis of the formyl phosphate supported reactions indicates that the low steady-state rates observed for the synthetic intermediate are most likely due to the sequential nature of the normal reaction.

Chemical activation of substrates by a nucleoside triphosphate dependent phosphorylation is believed to be an essential step in the mechanisms of several ligase enzymes, including that of N^{10}-formyltetrahydrofolate (N^{10}-formyl-H_4folate)[1] synthetase (EC 6.3.4.3) (Himes & Harmony, 1973). This enzyme catalyzes an ATP-dependent formylation of H_4-folate into N^{10}-formyl-H_4folate:

† This work was supported by Grants AM 17517/36842 and GM 35752 (G.H.R.) and Grant AM 07140 (R.H.H.) from the National Institutes of Health and by NRSA Fellowship GM 07229 (J.L.K.).

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[1] Abbreviations: H_4folate, tetrahydrofolate; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid (sodium salt); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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$$Mg^{II}ATP + H_4folate + formate \rightleftharpoons Mg^{II}ADP + P_i + N^{10}\text{-formyl-}H_4folate$$

Steady-state kinetic measurements (Joyce & Himes, 1966a; McGuire & Rabinowitz, 1978), together with negative findings from partial exchange reactions (Himes & Rabinowitz, 1962; Joyce & Himes, 1966b; McGuire & Rabinowitz, 1978), are consistent with a random sequential mechanism in which any intermediates in the reaction scheme must remain tightly bound at the active site.

The chemical lability of formyl phosphate (Jaenicke & Koch, 1963), the putative intermediate, has heretofore hampered a direct test for its presence and has also hindered studies on the substrate activity of the synthetic compound. Evidence for this species was originally based on the observation of ^{18}O transfer from formate to P_i during the forward enzymic reaction (Himes & Rabinowitz, 1962). Supporting evidence for the intermediate has been provided by the observation that the enzyme catalyzes synthesis of ATP from ADP and carbamoyl phosphate, a more stable structural analogue of formyl phosphate, in a reaction that requires H_4folate as a cofactor (Buttlaire et al., 1976, 1979). The reaction with carbamoyl phosphate, however, proceeds at a rate that is only 1% of that measured for the normal reverse reaction catalyzed by the synthetase.

Previous attempts to use synthetic formyl phosphate as a substrate for the enzyme have proved unsuccessful (Jaenicke & Brode, 1961; Sly & Stadtman, 1963). However, the details of these experiments were not published. The intrinsic lability of formyl phosphate (Jaenicke & Koch, 1963), and the possibility that ADP may be required as a cofactor in the reaction (Buttlaire et al., 1976), prompted a reinvestigation of the synthesis, stability, and substrate activity of this compound.

Experimental Procedures

Chemicals. Benzoyl chloride and KFHF were obtained from Alfa Products, and 2-mercaptoethanol, DSS, Ap_5A, and TMS were purchased from Sigma Chemical Co. Hepes and $H_2{}^{18}O$ (98 atom % ^{18}O) were purchased from Research Organics Inc. and Cambridge Isotope Laboratories, respectively. The lithium salt of ADPβS was obtained from Boehringer-Mannheim. (6*RS*)-H_4folate was prepared by the catalytic hydrogenation of folic acid and purified as described elsewhere (Samuel et al., 1970). A solution of N^5,N^{10}-methenyl-H_4folate was prepared by dissolution of N^5-formyl-H_4folate (Sigma Chemical Co.) in 0.24 M HCl. Pteroyltriglutamate was a gift from the laboratory of Dr. J. C. Rabinowitz (University of California, Berkeley, CA). Other chemicals were analytical reagent grade and were used without further purification.

Enzymes, Enzyme Assays, and Steady-State Kinetics. N^{10}-Formyl-H_4folate synthetase was isolated from *Clostridium cylindrosporum* as described previously (Buttlaire, 1980). The enzyme preparation had a specific activity of 350–400 IU/mg of protein at 37 °C, as measured in the spectrophotometric assay involving acid-catalyzed cyclization of N^{10}-formyl-H_4-folate into N^5,N^{10}-methenyl-H_4folate (λ_{max} = 350 nm) (Rabinowitz & Pricer, 1962). This assay was also used to monitor formation of N^{10}-formyl-H_4folate from formyl phosphate and H_4folate, in the forward direction of the reaction. Synthesis of ATP from formyl phosphate and ADP, in the reverse direction of the reaction, was measured in a coupled, spectrophotometric assay with hexokinase and glucose-6-phosphate dehydrogenase (mixed enzymes from bakers' yeast, Sigma Chemical Co.), essentially as described previously (Buttlaire et al., 1976). Enzyme assays with formyl phosphate as the substrate were carried out at pH 7 instead of pH 8 because formyl phosphate is less stable at the higher pH [see Table I and Jaenicke and Koch (1963)]. Steady-state kinetic results were analyzed by using a Hanes plot ([S]/v vs. [S]), and K_m values were obtained by linear regression analysis of the data. The k_{cat} values were determined from reciprocal plots of experimental data in which formyl phosphate and H_4folate (forward reaction) or formyl phosphate and ADP (reverse reaction) were varied in a constant ratio.

Table I: Hydrolysis of Formyl Phosphate[a]

solvent[b]	buffer concn[c] (M)	pH	temp (°C)	rate constant ($min^{-1} \times 10^2$)
H_2O		7.0	20	0.72
^2H_2O		7.0 (p^2H)	20	0.58
H_2O	0.1	7.0	20	1.4
^2H_2O	0.1	7.0 (p^2H)	20	0.69
H_2O	0.1	8.0	20	2.7
H_2O	0.1	7.0	30	2.9
^2H_2O	0.1	7.0 (p^2H)	30	2.2
H_2O	0.2	7.0	30	4.1
H_2O	0.5	7.0	30	7.7
H_2O	0.1	7.0	5	0.24
"assay mixture"	0.05	7.0	20	3.5
"assay mixture"	0.05	8.0	20	6.3

[a] Rates of hydrolysis were determined by using ^{31}P NMR (see Figure 1A) to follow the rate of increase in the area of the P_i resonance and the concomitant decrease in the area of the formyl phosphate peak. [b] The H_2O solvent contained 10% (v/v) ^2H_2O which served as the NMR field frequency lock. The "assay mixture" contained 50 mM Hepes, 40 mM Tris, 1 mM $MgCl_2$, 0.1 mM ADP, 100 mM 2-mercaptoethanol, and 2 mM (6*RS*)-H_4folate. [c] Buffer solutions contained Hepes at the indicated concentration and were adjusted to the required pH with NH_4OH.

Synthesis of Formyl Phosphate.[2] The lithium salt of formyl phosphate was prepared from formyl fluoride and inorganic phosphate, based on the procedure described by Jaenicke and Koch (1963). Formyl fluoride was synthesized from anhydrous formic acid and KFHF, in the presence of benzoyl chloride, by the method of Olah and Kuhn (1960). Formyl fluoride (bp −29 °C) was collected as a liquid in a trap immersed in a dry ice/ethanol bath. Gaseous formyl fluoride (equivalent to approximately 5 mL of liquid) was introduced slowly (through a gas dispersion tube with a fritted disk) into 50 mL of an ice-cold solution of 1 M K_2HPO_4 in 1 M $KHCO_3$, contained in a 100-mL graduated cylinder. Other steps were carried out as described by Jaenicke and Koch (1963), with the following exception. In the step in which excess phosphate was precipitated with $AgNO_3$, the pH of the solution was maintained at approximately 7 instead of 5–6. The higher pH resulted in a more efficient precipitation of silver phosphate without significantly jeopardizing the yield of formyl phosphate (see Table I). The white powder containing the product was stored dry, under reduced pressure, at −20 °C. Under these conditions, the compound is stable for at least several months.

Characterization of Formyl Phosphate. The formyl phosphate preparation gave a positive reaction with hydroxylamine and $FeCl_3$ in a colorimetric assay for acyl phosphates (Stadtman, 1957).

The ^{31}P NMR spectrum (121.4 MHz) of the product (Figure 1A) shows three sets of resonances: a doublet (δ 0.53, $^3J_{PH}$ = 3.3 Hz) assigned to formyl phosphate and singlet resonances due to P_i (δ 2.70) and PP_i (δ −5.61). The presence of PP_i was confirmed by the addition of authentic PP_i to the

[2] Benzoyl chloride is a lachrymator, and formyl fluoride is a severe irritant. Hydrofluoric acid may also be present at various stages of this synthesis. Appropriate precautions should be exercised in handling these materials.

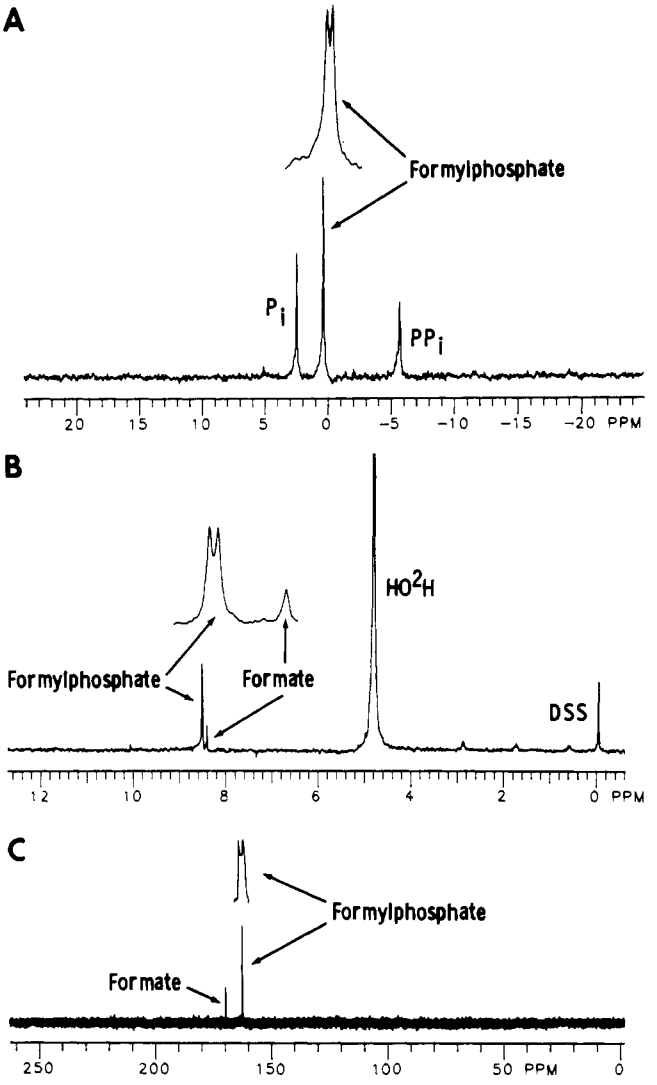

FIGURE 1: (A) ^{31}P NMR spectrum (121.4 MHz) of formyl phosphate (12 mg/mL) dissolved in ^2H_2O (p^2H 7.0). Spectrometer conditions included the following: sweep width, 6000 Hz; pulse width, 17.3 μs (26° tip angle); number of transients, 50. Expansion (15×) of the downfield doublet resonance (J = 3.3 Hz) is shown. Chemical shifts are reported with reference to 85% H_3PO_4; T = 20 °C. (B) ^1H NMR spectrum (300 MHz) of formyl phosphate (12 mg/mL) dissolved in ^2H_2O (p^2H 7.0). Spectrometer conditions included the following: sweep width, 5000 Hz; pulse width, 5.0 μs (16° tip angle); number of transients, 100. Expansion (15×) of the downfield doublet resonance (J = 3.3 Hz) is shown. Chemical shifts are reported with reference to the methyl resonance of DSS; T = 20 °C. (C) Proton-decoupled ^{13}C NMR spectrum (75 MHz) of formyl phosphate (30 mg/mL) dissolved in ^2H_2O (p^2H 7.0). Spectrometer conditions included the following: sweep width, 20000 Hz; pulse width, 8.9 μs (54° tip angle); number of transients, 2500. Expansion (28×) of the downfield doublet resonance (J = 2.4 Hz) is shown. Chemical shifts are reported with reference to the methyl resonance of TMS; T = 7 °C.

sample. The ^1H NMR spectrum (300 MHz) of the preparation (Figure 1B) shows a singlet resonance (δ 8.44) due to formate and a doublet signal (δ 8.52, $^3J_{HP}$ = 3.3 Hz) assigned to formyl phosphate. The concentration of formyl phosphate in the solution was estimated from the area of the ^1H NMR signal, relative to that of the methyl groups of DSS, added as an internal standard. The natural-abundance, proton-decoupled ^{13}C NMR spectrum (75 MHz) of the preparation (Figure 1C) shows a singlet resonance due to formate (δ 169.7) and a doublet signal assigned to formyl phosphate (δ 162.5, $^2J_{CP}$ = 2.4 Hz).

^{31}P NMR was used to measure the stability of formyl phosphate. Results of this study are presented in Table I.

Table II: Substrate Activity of Formyl Phosphate in the Forward Reaction Catalyzed by N^{10}-Formyl-H_4folate Synthetase

reaction mixture[a]	N^5,N^{10}-methenyl-H_4folate[b] (nmol)	
	30 °C, 30 min	20 °C, 2 min
complete (10 μg of enzyme)	46 ± 4	
complete (10 μg of enzyme + 100 μM Ap_5A)	44 ± 4	
complete (5 μg of enzyme)	34 ± 3	
complete (2.5 μg of enzyme)	22 ± 2	
complete (75 μg of enzyme)		23
complete (50 μg of enzyme)		17
complete (25 μg of enzyme)		11
minus enzyme	0	0
minus ADP	0	0
minus ADP (+2 mM ADPβS)	9	2
minus formyl phosphate	0	0
minus $MgCl_2$ (+2 mM EDTA)	0	
minus formyl phosphate (+150 mM NH_4HCO_2)[c]	0	

[a] Complete reaction mixtures contained 50 mM Hepes/NH_3, pH 7.0, 10 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 2.0 mM ADP, 1.0 mM (6*RS*)-H_4folate, 10 mM formyl phosphate, and enzyme in 0.5 mL. Mixtures lacking reaction components contained 10 μg (30 °C, 30 min) or 50 μg (20 °C, 2 min) of enzyme. Reactions were terminated with 1.0 mL of 0.36 M HCl. Averaged values are from duplicate determinations. [b] Acid-catalyzed cyclization product of N^{10}-formyl-H_4folate (ϵ_{350} = 24.9 mM^{-1} cm^{-1}). [c] Control assay for adenylate kinase.

Formyl phosphate is most stable at low temperature in solutions of neutral pH and low buffer concentration. A substantial solvent deuterium isotope effect was also noted in the rate of hydrolysis of formyl phosphate dissolved exclusively in ^2H_2O. The half-life of formyl phosphate dissolved in the "assay mixture" at pH 7 and 20 °C (20 min) (Table I) indicates that the synthetic compound has sufficient stability under these conditions to probe the chemical mechanism of N^{10}-formyl-H_4folate synthetase.

The position of bond cleavage upon hydrolysis of formyl phosphate was investigated by incubation of the compound in $H_2{}^{18}O$ under neutral, acidic, and alkaline conditions and subsequent analysis of the reaction products by ^{31}P NMR. Spectra for the products of hydrolyses carried out at pH 4, 7, and 9 exhibited signals for the $[^{16}O_4]P_i$ contaminant of the starting material, and possibly $[^{16}O_4]P_i$ resulting from C–O bond cleavage, as well as a signal 0.02 ppm upfield from P_i due to the $[^{16}O_3,{}^{18}O]P_i$ species, produced by P–O bond cleavage. Analysis of the areas under the two peaks showed that approximately 90% of the formyl phosphate was hydrolyzed by P–O bond cleavage at pH 7. Further studies using ^{13}C NMR are planned to determine whether any C–O bond cleavage occurs at this pH value. By contrast, spectra for the products of hydrolyses carried out at pH 1 and 13 showed a single resonance for P_i, consistent with C–O bond cleavage of formyl phosphate under these conditions. Thus, the mechanism for hydrolysis of formyl phosphate appears to be analogous to that reported earlier for acetyl phosphate (Koshland, 1952).

Results

Assays for Production of N^{10}-Formyl-H_4folate. Formyl phosphate serves as a substrate in the forward direction of the synthetase-catalyzed reaction. Results of activity assays and various control experiments are summarized in Table II. Incubation of reaction mixtures that contained formyl phosphate, enzyme, Mg(II), ADP, and H_4folate led to formation of N^{10}-formyl-H_4folate, as evidenced by the absorption spectrum following acid treatment of the mixture.[3] The spectrum

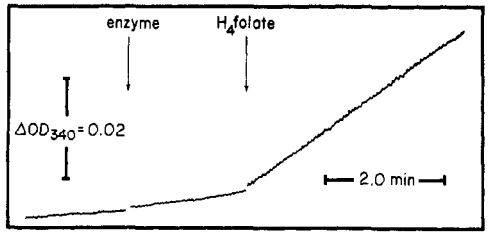

FIGURE 2: Coupled assay showing ATP synthesis from formyl phosphate and ADP in the reverse reaction catalyzed by N^{10}-formyl-H_4folate synthetase, as measured by $NADP^+$ reduction at 340 nm. The reaction mixture contained 50 mM Hepes/NH_3, pH 7.0, 1 mM $MgCl_2$, 2 mM ADP, 100 mM 2-mercaptoethanol, 1 mM D-glucose, 0.25 mM $NADP^+$, 5 mM formyl phosphate, 13.3 IU of hexokinase, 5 IU of glucose-6-phosphate dehydrogenase, 36.4 μg of N^{10}-formyl-H_4folate synthetase, and 1.0 mM (6*RS*)-H_4folate in a final volume of 1.0 mL. The synthetase enzyme and H_4folate were added at the points indicated by the arrows; T = 20 °C.

(330–450 nm, λ_{max} = 350 nm) matched that for authentic N^5,N^{10}-methenyl-H_4folate.

The cofactor requirements for Mg(II) and, in particular, ADP (see Table II), together with the presence of formate in the preparation of formyl phosphate (see Figure 1B,C), necessitated control experiments to eliminate the possibility that the observed activity was due to ATP, produced by an adenylate kinase contamination of the synthetase preparation. Three lines of experimental evidence argue against any influence of adenylate kinase in the observed substrate activity of formyl phosphate. First, the activity is independent of the presence of the inhibitor of adenylate kinase, Ap_5A (Table II). Second, no activity is found for a reaction mixture containing enzyme, Mg(II), H_4folate, formate, and ADP (Table II). Finally, incubation of a reaction mixture containing Mg(II), ADP, and enzyme ($\simeq$1 mg) at 37 °C for 5 h resulted in no detectable ATP formation, as assessed by HPLC. These control experiments rule out complications from adenylate kinase.

ATP (and formate) could also be produced from formyl phosphate and ADP in a reaction catalyzed by formate kinase. Such activity has been implicated as a minor contaminant in some preparations of N^{10}-formyl-H_4folate synthetase (Sly & Stadtman, 1963). In order to test for this activity, reaction mixtures that lacked H_4folate were incubated for 30 min at 30 °C. Aliquots of the mixture were analyzed for ATP by HPLC. Under these conditions, 4 ± 1 nmol of ATP was formed. The amount of ATP produced in the absence of H_4folate is not sufficient to account for the quantity of product formed in the complete reaction mixture (see Table II).

The reversible inactivation of N^{10}-formyl-H_4folate synthetase by the removal of specific monovalent cations (Himes & Harmony, 1973) eliminated ATP production with or without H_4folate present. It is unlikely that formate kinase or adenylate kinase would be similarly inactivated by this procedure. ATP synthesis in the absence of H_4folate can be attributed to a slow, cofactor-independent, synthetase-catalyzed phosphorylation of ADP (see below and Figure 2).

Assays for Production of ATP. Formyl phosphate also supports the formation of ATP from ADP, in the presence of enzyme, Mg(II), and H_4folate. Results of a coupled assay, in which $NADP^+$ reduction is followed at 340 nm, are illustrated in Figure 2. The very slow background rate, measured in the absence of H_4folate, was markedly enhanced by addition of the reduced folate cofactor. Conversely, in the absence of the synthetase enzyme, addition of H_4folate did not influence the initial background rate. The substantial stimulation of ATP synthesis concomitant with the addition of H_4folate (Figure 2) indicates that the synthetase enzyme, and not adenylate kinase or formate kinase contamination, must be responsible for the vast majority of the observed ATP production. Moreover, the observed changes in absorbance were insensitive to the presence of the adenylate kinase inhibitor, Ap_5A (100 μM). ATP synthesis was also stimulated by pteroyltriglutamate, a competitive inhibitor of H_4folate (K_i = 0.83 mM). At equal concentrations (0.2 mM), the stimulated rate in the presence of the triglutamate analogue was 20% of that observed with H_4folate.

Table III: Steady-State Kinetic Constants for the Forward and Reverse Reactions Catalyzed by N^{10}-Formyl-H_4folate Synthetase

varied substrate/ cofactor	K_m (mM)		k_{cat} (s^{-1})	
	forward	reverse	forward	reverse
(A) Reactions with Formyl Phosphate[a]				
formyl phosphate	3.4 ± 1.2	3.1 ± 1.1	5.1 ± 0.9	0.16 ± 0.04
(6*RS*)-H_4-folate	0.51	0.25		
ADP	0.04	0.04		
ADPβS	0.02			
(B) Reactions with Carbamoyl Phosphate[b]				
carbamoyl phosphate	11.9 (10)[c]			0.8 (0.13)[c]
(6*RS*)-H_4-folate	0.06–0.16			
ADP	0.03			
(C) Normal Reactions[d]				
formate	8.3		650 (150 ± 10)[c]	80
(6*RS*)-H_4-folate	0.74			
ADP	0.13			

[a] T = 20 °C, pH 7.0. Assay conditions are described in Table II (20 °C, 2 min) (forward reaction) and in the legend to Figure 2 (reverse reaction). [b] T = 37 °C, pH 8.0. Data taken from Buttlaire et al. (1976, 1979). [c] Values in parentheses were determined in the present study. T = 20 °C, pH 7.0. [d] T = 37 °C, pH 8.0. Data taken from Himes and Harmony (1973).

Steady-State Kinetics. Results of a steady-state kinetic analysis of the forward and reverse reactions catalyzed by N^{10}-formyl-H_4folate synthetase with formyl phosphate are presented in Table III. Kinetic parameters for the reverse reaction catalyzed by the synthetase with carbamoyl phosphate, as well as data for the normal forward and reverse reactions, are also given in Table III.

K_m values for ADP and for H_4folate in the formyl phosphate supported reactions compare favorably with values for the normal reaction and also with values obtained for the carbamoyl phosphate supported reverse reaction (Table III). The k_{cat} value for the formyl phosphate supported reverse reaction is comparable to that for the analogous reaction with carbamoyl phosphate (Table III). Although formyl phosphate satisfies the chemical requirements for production of N^{10}-formyl-H_4folate in the forward reaction (Table II), and of ATP in the reverse reaction (Figure 2), the apparent k_{cat} values for the synthetic intermediate are only approximately 3–4% (forward reaction) and 1% (reverse reaction)[4] of those for the normal reactions (Table III).

Hydrolysis of formyl phosphate results in the production of formate and P_i, and varying quantities of these breakdown

[3] Negligible hydrolysis of formyl phosphate occurred during the course of the reaction, when carried out at 20 °C for 2 min (see Table II). The half-life of the substrate under these conditions is 20 min (see Table I).

[4] Based on the assumption that k_{cat} for the normal reverse reaction at 20 °C is 20% of that at 37 °C (see Table III).

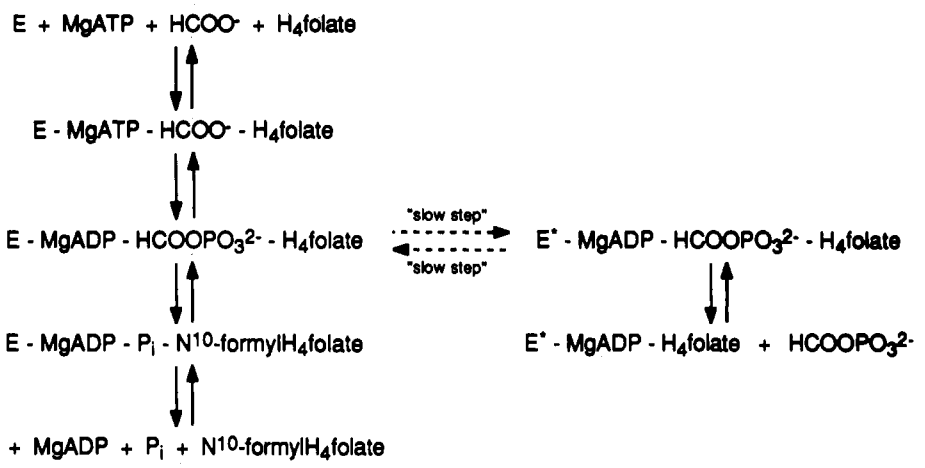

FIGURE 3: Sequential reaction scheme for enzyme-bound substrates, depicted with formyl phosphate ($HCOOPO_3^{2-}$) as the "nondissociable" intermediate, for the reaction catalyzed by N^{10}-formyl-H_4folate synthetase. The reaction of free formyl phosphate with the dead-end complex, [enzyme–Mg^{II}ADP–H_4folate], is shown as a side pathway. An isomerization of the enzyme (E to E*) represents the kinetic barrier to dissociation of formyl phosphate in the normal catalytic cycle. Charges on ATP, ADP, and H_4folate have been omitted for simplicity.

products, together with PP_i, are present in all preparations of the compound (see Figure 1). The relatively low steady-state rates measured for the reactions with formyl phosphate (Table III) are, however, not attributable to strong inhibition by these contaminants. Results from a detailed kinetic study of the carbamoyl phosphate supported reverse reaction show that both formate (K_i = 60 mM) and P_i (K_i = 2–12 mM) are weak binding inhibitors (Buttlaire et al., 1979). Thus, inhibition by formate is negligible based on the low level of this contaminant in the preparations of formyl phosphate (see Figure 1B) and the high K_i for formate. Contamination by inorganic phosphate and pyrophosphate varied with each formyl phosphate preparation (e.g., PP_i contamination varied between 5% and 25% of the concentration of formyl phosphate). The rates obtained from different preparations, however, varied by, at most, a factor of 2. Furthermore, pretreatment of an assay mixture with inorganic pyrophosphatase (10 units for 5 min) enhanced the rate with formyl phosphate only 2-fold. Thus, while formate, P_i, and PP_i are weak inhibitors, their actual concentrations in the reaction mixtures are not sufficient to account for the 30–100-fold differences between the k_{cat} values, respectively, for the normal forward and reverse reactions, and those obtained with synthetic formyl phosphate (Table III).

The possibility that the steady-state rates with formyl phosphate are lowered by some other inhibitory species in the preparation has been explored. Potential inhibitory species that might be carried over during the synthesis and workup of formyl phosphate have either been assayed in the preparation or tested for inhibitory effects in both the normal reaction and carbamoyl phosphate supported reverse reaction.[5] Any traces of Ag^+ would be effectively masked by the high concentration of 2-mercaptoethanol used in the assays. Thus, salts of nitrate, perchlorate, and fluoride represent the only other species that might coprecipitate with formyl phosphate during the workup of the product. The "brown-ring" test for nitrate and elemental analysis for nitrogen in the preparations were negative. $LiClO_4$ and KF at concentrations of 4 mM did not inhibit either the normal forward reaction of the enzyme or the reaction with carbamoyl phosphate. The possibility that the enzyme was inactivated by reaction with formyl phosphate under assay conditions was also eliminated. Enzyme that had been preincubated with formyl phosphate showed normal activity in the forward direction of the synthetase assay. Although the k_{cat} values measured for formyl phosphate (Table III) may be lowered slightly due to weak inhibition by P_i and PP_i, saturation kinetics found for formyl phosphate, as well as exhaustive screening for other inhibitors in the preparation, indicate that the intrinsic k_{cat} values for exogenous formyl phosphate are indeed low relative to those of the normal reaction.

Discussion

A scheme for the reaction of enzyme-bound substrates and products, depicted with formyl phosphate as an intermediate, is shown in Figure 3. Results of the present experiments demonstrate that formyl phosphate is indeed a substrate for N^{10}-formyl-H_4folate synthetase in both the forward and reverse directions. From the chemical perspective, this compound could certainly be an intermediate in the normal reaction catalyzed by the enzyme. Although formyl phosphate obviously does not satisfy the criterion for kinetic competence of a freely dissociable intermediate, the kinetic data neither affirm nor negate the existence of formyl phosphate as a nondissociable intermediate in the normal catalytic cycle.

In a sequential mechanism, the labile intermediate is conserved by sticking at the active site in a nondissociable manner. Two possible strategies for trapping the intermediate at the active site are as follows: (1) a low dissociation constant for the intermediate (i.e., high thermodynamic stability for the complex with the intermediate); or (2) a slow dissociation of the intermediate coupled with a slow binding step (i.e., a kinetic trap of the intermediate). High thermodynamic stability for the intermediate complex with formyl phosphate would diminish the reactivity of the intermediate because the probable transition states for the reactions flanking the complex with formyl phosphate require stabilization of different geometries,[6] either at the phospho or at the carboxyl centers. By contrast, substantial kinetic barriers to dissociation and productive binding accomplish conservation of the labile intermediate without compromising appreciably the desired reactivity of the activated compound. Further, the unfavorable barrier to binding is never encountered during the normal reaction se-

[5] The reaction with carbamoyl phosphate involves the same reaction mixture (i.e., the dead-end complex of product and substrate) as in assay mixtures for the reactions with formyl phosphate.

[6] The transition state for formation of formyl phosphate in the forward direction likely involves a trigonal bipyramidal geometry about the phospho group, and the transition state for formyl transfer from formyl phosphate to H_4folate likely involves a pseudotetrahedral geometry about the carboxyl center of formyl phosphate.

quence wherein the activated intermediate is generated in situ (Figure 3). The strategy involving kinetic barriers, therefore, has advantages for catalytic efficiency.

A minimal scheme that fits the steady-state kinetic data for exogenous formyl phosphate is shown as a side pathway in Figure 3. The observation of saturation kinetics for the reactions with exogenous formyl phosphate requires that the initial binding step not be rate limiting. Hence, free formyl phosphate combines reversibly with the enzyme–Mg^{II}ADP–H_4folate complex, and the K_m values for formyl phosphate relate to this association. There must, however, be a "slow step" (i.e., E* to E) prior to reaction in either direction that is the reverse of the required isomerization that occurs prior to release of the formyl phosphate intermediate. This slow step represents the kinetic barrier to "productive binding" of the intermediate (Figure 3). Thus, the low k_{cat} values for the forward and reverse directions of the reaction with exogenous formyl phosphate, relative to the k_{cat} values for the normal forward and reverse reactions, reflect the "slow binding" aspect of the kinetic strategy for holding the intermediate at the active site. Subsequent to this slow step, the reaction partitions between the forward and reverse directions according to the respective conductances through these pathways (Cleland, 1975).

The k_{cat} for the carbamoyl phosphate dependent production of ATP (Buttlaire et al., 1976, 1979) is comparable to that observed for the analogous reaction with formyl phosphate. In light of the conclusions regarding a required slow step in the reaction with formyl phosphate, it appears that carbamoyl phosphate is, in fact, an excellent analogue of formyl phosphate in this reaction.

The typically fleeting existence of putative intermediates for sequential enzymic reactions has resulted in a sparsity (or absence) of information on the steady-state kinetic behavior of corresponding synthetic compounds. Formyl phosphate is one of the more stable proposed intermediates for such reactions, and it may be possible to trap and to identify this intermediate using rapid quench methods.

ACKNOWLEDGMENTS

We thank Drs. W. W. Cleland and R. L. Schowen for helpful discussions.

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