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Formation and Utilization of Formyl Phosphate by N^{10}-Formyltetrahydrofolate Synthetase: Evidence for Formyl Phosphate as an Intermediate in the Reaction†

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Received January 31, 1989; Revised Manuscript Received March 14, 1989

ABSTRACT: N^{10}-Formyltetrahydrofolate synthetase from bacteria and yeast catalyzes a slow formate-dependent ADP formation in the absence of H_4folate. The synthesis of formyl phosphate by the enzyme was detected by trapping the intermediate as formyl hydroxamate. That the "formate kinase" activity was part of the catalytic center of N^{10}-formyltetrahydrofolate synthetase was shown by demonstrating coordinate inactivation of the "kinase" and synthetase activities by heat and a sulfhydryl reagent, similar effects of monovalent cations, similar K_m values for substrates, and similar K_i values for the inhibitor phosphonoacetaldehyde for both activities. The relative rates of the kinase activities for the bacterial and yeast enzymes are about 10^{-4} and 4×10^{-6} of their respective synthetase activities. These slow rates for the kinase reaction can be explained by the slow dissociation of ADP and formyl phosphate from the enzyme. This conclusion is supported by rapid-quench studies where a "burst" of ADP formation (6.4 s^{-1}) was observed that is considerably faster than the steady-state rate (0.024 s^{-1}). The demonstration of enzyme-bound products by a micropartition assay and the lack of a significant formate-stimulated exchange between ADP and ATP provide further evidence for the slow release of the products from the enzyme. The synthesis of N^{10}-CHO-H_4folate when H_4folate was added to the E-formyl phosphate-ADP complex is also characterized by a "burst" of product formation. The rate of this burst phase at 5 °C occurs with a rate constant of 18 s^{-1} compared to 14 s^{-1} for the overall reaction at the same temperature. These results provide further evidence for formyl phosphate as an intermediate in the reaction and are consistent with the sequential mechanism of the normal catalytic pathway. Positional isotope exchange experiments using [β,γ-^{18}O]ATP showed no evidence for exchange during turnover experiments in the presence of either H_4folate or the competitive inhibitor pteroyltriglutamate. The absence of scrambling of the ^{18}O label as observed by ^{31}P NMR suggests that the central complex may impose restraints to limit free rotation of the P_β oxygens of the product ADP.

The enzyme N^{10}-formyltetrahydrofolate synthetase catalyzes the ATP-dependent formylation of H_4folate[1] at the N^{10}-position (eq 1). The procaryotic enzyme is a monofunctional

$$\text{MgATP} + \text{HCOO}^- + \text{H}_4\text{folate} \overset{M^+}{\leftrightarrow} \text{MgADP} + \text{P}_i + N^{10}\text{-HCO-H}_4\text{folate} \quad (1)$$

tetramer of four identical subunits (Himes & Harmony, 1973). In eucaryotic organisms, the N^{10}-HCO-H_4folate synthetase activity along with N^5,N^{10}-methenylH_4folate cyclohydrolase and N^5,N^{10}-methyleneH_4folate dehydrogenase activities exists as a dimeric, multifunctional, and multidomain protein called C_1-H_4folate synthase. Properties of these enzymes are described in two review articles (Himes & Harmony, 1973; MacKenzie, 1984).

Most of the previous studies on the mechanism of the reaction have been done with the bacterial enzyme. Steady-state

† This work was supported by National Institutes of Health Grants DK-071410 (R.H.H.) and AR-17323 (G.L.K.) and grants from the University of Kansas General Research Fund and Biomedical Research Support Funds.

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[1] Abbreviations: H_4folate, tetrahydrofolate; N^{10}-HCO-H_4folate, N^{10}-formyltetrahydrofolate; $PteGlu_3$, pteroyltriglutamate; Pte, pteroic acid; TFA-Pte, (trifluoroacetyl)pteroic acid; TFA-$PteGlu_3$, [(trifluoroacetyl)pteroyl]triglutamate; *t*-Boc-Glu-γ-OBzl, *tert*-butoxycarbonyl-L-glutamic acid γ-benzyl ester; HOBt, hydroxybenzotriazole; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/89/0428-5136$01.50/0

kinetic and isotope exchange data (Joyce & Himes, 1966a,b; McGuire & Rabinowitz, 1978) were consistent with a random sequential mechanism for the reaction. Various types of experimental approaches ruled out the formation of freely dissociable intermediates, and a concerted mechanism was proposed (Himes & Rabinowitz, 1962b). The formation of a tightly bound intermediate was not ruled out by the data, however. Several forms of evidence have gradually emerged to support the proposal that formyl phosphate is an enzyme-bound intermediate. Indirect proof came from the demonstration of a carbamoyl phosphate dependent phosphorylation of ADP requiring H_4folate as a cofactor (Buttlaire et al., 1976, 1979). More recent evidence was the finding that chemically synthesized formyl phosphate can support the forward reaction by formylating H_4folate (eq 2) and the reverse reaction by phosphoryl transfer to ADP (eq 3) (Smithers et al., 1987).

$$HCOOPO_3^{2-} + H_4\text{folate} \overset{ADP}{\leftrightarrow} N^{10}\text{-HCO-}H_4\text{folate} + P_i \quad (2)$$

$$HCOOPO_3^{2-} + ADP \overset{H_4\text{folate}}{\leftrightarrow} ATP + HCOO^- \quad (3)$$

The requirements for ADP and H_4folate as cofactors in the formyl phosphate supported reactions were in agreement with previous kinetic (Joyce & Himes, 1966a,b; McGuire & Rabinowitz, 1978) and spectroscopic data (Buttlaire et al., 1975a,b) that a full complement of substrates is needed to produce a catalytically functional structure. The existence of formyl phosphate as an intermediate in the reaction is also supported by the finding that ^{18}O is transferred from formate to inorganic phosphate during the forward reaction (Himes & Rabinowitz, 1962b).

These previous studies, however, do not provide unequivocal evidence that formyl phosphate is actually formed during the reaction but rather simply suggest that the catalytic center supports formyl or phosphoryl transfer from formyl phosphate (or its analogue) to an acceptor molecule. One purpose of this study was to demonstrate the formation of this putative enzyme-bound intermediate by use of the enzyme from bacteria and yeast, i.e., to show a "formate kinase" activity (eq 4).

$$HCOO^- + ATP \leftrightarrow HCOOPO_3^{2-} + ADP \quad (4)$$

Another purpose was to obtain evidence that formyl phosphate is a kinetically competent intermediate in the overall reaction.

Experimental Procedures

Preparation of the Enzymes. N^{10}-HCO-H_4folate synthetase was prepared from *Clostridium cylindrosporum* according to a previously published procedure (Mejillano & Himes, 1986). The enzyme was further purified by DEAE-cellulose chromatography according to the method of Buttlaire (1980). The eucaryotic enzyme from *Saccharomyces cerevisiae* was isolated and purified by heparin–agarose and matrex gel red A chromatography according to the procedure of Staben et al. (1987). The strain of *S. cerevisiae* (provided by Dr. Staben, University of California) had been transformed with the YEpADE3 plasmid. The bacterial N^{10}-HCO-H_4folate synthetase assay was performed according to the procedure of Rabinowitz and Pricer (1962). The assay mixture for the yeast enzyme was the same as that reported earlier (Staben et al., 1987). In the assays, the N^{10}-HCO-H_4folate that is produced is converted to N^5,N^{10}-methenylH_4folate under acidic conditions, and the concentration of this product is determined spectrophotometrically at 350 nm (ϵ = 24 900 M^{-1} cm^{-1}).

Preparation of the Bacterial Monomer. A crystalline suspension of the bacterial enzyme in 50 mM potassium phosphate, pH 7.5, 50 mM 2-mercaptoethanol, and 50% $(NH_4)_2SO_4$ was collected by centrifugation, and the pellet was dissolved in 200 μL of 25 mM Tris-HCl, pH 8, and 50 mM 2-mercaptoethanol. The enzyme solution was then centrifuged through 1 mL of Sephadex G-25 in a 1-mL syringe in the same buffer. The monomer prepared in this manner did not show any detectable activity.

Synthesis of Pteroyltriglutamate. Pteroyltriglutamate ($PteGlu_3$) was synthesized according to the solid-phase peptide method by coupling (trifluoroacetyl)pteroic acid to the triglutamyl resin. N^{10}-(Trifluoroacetyl)pteroic acid (TFA-Pte) was synthesized chemically from trifluoroacetic acid and pteroic acid by the method of Godwin et al. (1972). Pteroic acid was produced by a fermentative method by removal of glutamic acid from commercial-grade pteroylglutamic acid (folic acid) with a folic acid degrading *Pseudomonas* species (Levy & Goldman, 1967; Goldman & Levy, 1971) and purified by chromatography on cellulose CF11 (Whatman) (Scott, 1980).

The procedures employed in the synthesis of $PteGlu_3$ were modifications of the Merrifield solid-phase peptide synthesis method employed by Krumdieck and Baugh (1969) for the synthesis of pteroylglutamic acid derivatives. The incorporation of the first glutamic acid residue was done by esterification of the chloromethylated resin (Peninsula) with *t*-Boc-Glu-γ-OBzl according to the method of Horiki et al. (1978). The next steps of deprotection, neutralization, and coupling of the γ-carboxyl end of the second and third glutamic acid residues and of TFA-Pte to the resin were done on a manual solid-phase peptide synthesizer (Peninsula) according to a standard protocol described by Stewart and Young (1984). The coupling reactions utilized the ester of hydroxybenzotriazole (HOBt), which was prepared separately before addition to the resin by mixing 3 equiv each of HOBt, dicyclohexylcarbodiimide, and *t*-Boc-Glu-α-OBzl or the protected pteroic acid per equivalent of resin. The TFA-$PteGlu_3$ was cleaved from the resin with hydrogen bromide in acetic acid. From the absorbance of the crude product in 0.1 M NaOH at 365 nm (ϵ = 9260 M^{-1} cm^{-1}) (Krumdieck & Baugh, 1969), a yield of 80% based on the starting TFA-Pte was obtained after cleavage from the resin. The removal of the trifluoroacetyl group was accomplished with piperidine as described by D'Ari and Rabinowitz (1985). The deprotected product was purified on a DEAE-Sephadex A-25 column with a salt gradient of 0.1–0.8 M NaCl in 5 mM potassium phosphate, pH 7.0. The purity of the compound was verified by TLC on cellulose plates (D'Ari & Rabinowitz, 1985) and by anion-exchange HPLC (Cashmore et al., 1980).

Synthesis of Phosphonoacetaldehyde. The lithium salt of phosphonoacetaldehyde was prepared from vinyl chloride, phosphorus pentachloride, and sulfur dioxide as described in the literature (Lutzencko & Kirilov, 1960; Isbell & Englert, 1969; LaNauze et al., 1977). The crude product was extracted twice with diethyl ether and twice with petroleum ether to remove the organic impurities. The purified product was precipitated with acetone, and its purity was determined by ^1H and ^{31}P NMR.

Assays of the Formate Kinase Activity. The reaction mixture for the bacterial enzyme contained 100 mM triethanolamine hydrochloride, pH 8, 5 mM ATP, 40 mM sodium formate, pH 8, 50 mM KCl, 10 mM $MgCl_2$, and 10–100 μM enzyme sites. The assay mixture for the yeast enzyme consisted of 25 mM K^+-Hepes, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 100 mM ammonium formate, and 30–50 μM enzyme sites. Reactions were done at 37 °C. For controls, formate was omitted from the reaction mixture to test for a possible ATPase activity or the enzyme was omitted to de-

termine the extent of ATP chemical hydrolysis under the assay conditions used. These controls produced an almost negligible activity.

The ADP produced was assayed by two methods: ion-exchange HPLC and an enzymatic assay. For the HPLC assay, aliquots of the reaction mixture were taken at different time intervals, diluted 3-fold in 0.5 M $NH_4H_2PO_4$, pH 4.3, filtered on nylon filters, and injected into a Partisil 10 SAX anion-exchange HPLC column (4.6 mm × 25 cm). Chromatography was performed at a flow rate of 1.0 mL/cm with a linear gradient of 0.01–0.75 M $NH_4H_2PO_4$, pH 4.3. The nucleotides were detected by their absorbance at 254 nm, and the concentration of ADP was determined from a calibration curve with standard ADP solutions or by comparison of the area of the ADP peak to the area of the peak representing the original ATP. The areas of the HPLC peaks were measured with a Sigma Scan computer program (Jandell Scientific).

In the enzyme assay for ADP, 0.1-mL aliquots of the reaction mixture taken at different time periods during the incubation were added to 0.9 mL of the assay mixture, which consisted of 100 mM triethanolamine hydrochloride, pH 8, 10 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, 1 mM phosphoenolpyruvate, pyruvate kinase (7 units/mL), and lactate dehydrogenase (10 units/mL) at 37 °C. The disappearance of NADH was monitored from the decrease in the absorbance at 340 nm, and the concentration of ADP was calculated with an ϵ value of 6.2 mM^{-1} cm^{-1} for NADH. The 10-fold dilution of the formate kinase assay components reduced the rate of the reaction to a rate that was undetectable in the time course of the ADP assay.

Formyl phosphate formation was assayed colorimetrically by its reaction with hydroxylamine to form the hydroxamate followed by complexation with Fe^{3+} (Sly & Stadtman, 1963). Neutral hydroxylamine (0.2 M) was incubated with the formate kinase reaction mixture. To 0.1 mL of the reaction mixture, 0.1 mL of 0.74 M trichloroacetic acid was added to precipitate the enzyme, and 0.2 mL of 0.22 M $FeCl_3$ in 1 M HCl was added to the supernatant after centrifugation to form the colored hydroxamate complex. The absorbance of the ferric hydroxamate at 505 nm was read within 30 min. The calibration curve for formyl hydroxamate was prepared from chemically synthesized formyl phosphate prepared from formyl fluoride and inorganic phosphate (Smithers et al., 1987). The purity of synthesized formyl phosphate was determined by ^1H NMR on the basis of a 3-(trimethylsilyl)-1-propanesulfonic acid standard.

Rapid-Quench Experiments. The formate kinase reaction was monitored at 37 °C at reaction times faster than 5 s on a Durrum multimixing apparatus. A 0.2-mL solution containing 100 μM triethanolamine hydrochloride, pH 8, 10 mM $MgCl_2$, 50 mM KCl, and 150 μM equivalent enzyme sites was added to an equal volume of solution consisting of 100 mM triethanolamine hydrochloride, pH 8, 10 mM $MgCl_2$, 50 mM KCl, 80 mM formate, and 10 mM [^3H]ATP (2×10^6 cpm/μmol). At various times, the reaction was quenched with 0.4 mL of 0.36 M HCl. The amount of [^3H]ADP formed was assayed by anion-exchange HPLC as described above. The fractions corresponding to ADP and ATP were collected, and the radioactivity was measured. A blank contained everything except the enzyme.

Similarly, the "burst" kinetics of the formyl transfer step from formyl phosphate to H_4folate was also studied at 37 and 5 °C for the multimixing apparatus for reaction times faster than 3 s or by manual mixing for longer times. A solution (0.2 mL) containing 100 mM triethanolamine hydrochloride, pH 8, 10 mM $MgCl_2$, 50 mM KCl, 4 mM ADP, 20 mM formyl phosphate, and 150 μM enzyme sites was first preincubated for 1 min and then was rapidly combined with 0.2 mL of a solution containing 100 mM triethanolamine hydrochloride, pH 8, 10 mM $MgCl_2$, 50 mM KCl, and 4 mM (*R,S*)-H_4folate. At varying time intervals, 0.4 mL of 0.36 M HCl was used to quench the reaction. After 10 min, the mixture was diluted, and the absorbance at 350 nm was measured. As a blank, the same solutions without enzyme were mixed rapidly and quenched with HCl after the same time periods. To test for the presence of contaminating ATP in ADP that may react with the formate contaminant in formyl phosphate and with H_4folate to give the same product, formate was added instead of formyl phosphate. There was a small but insignificant formation of N^{10}-HCO-H_4folate under these conditions.

The rate constant for the transient burst phase (λ) was evaluated on the basis of the method of Shafer et al. (1972) with

$$P = k_{ss}Et + \beta(1 + e^{-\lambda t}) \quad (5)$$

where P is the concentration of the product at time t, k_{ss} is the steady-state rate constant, E is the total enzyme concentration, and β is the transient burst amplitude.

Demonstration of Enzyme-Bound Products. The formation of enzyme-bound ADP and formyl phosphate was demonstrated by an ultrafiltration method using an Amicon centrifree micropartition apparatus with a YMT ultrafiltration membrane. This system separates protein-bound ligands which stay in the retentate (top reservoir) while the free ligands partition equally between the retentate and filtrate (lower reservoir).

The formate kinase reaction mixture (0.5 mL) containing either [^3H]ATP (5×10^7 cpm/μmol) or [^3H]formate (1×10^6 cpm/μmol) was incubated with the enzyme (60–250 μM sites) at 37 °C for 10 s. The solution was placed on ice and centrifuged into the micropartition apparatus at 4 °C for 3 min, and the retentate and filtrate portions were collected. The protein in the retentate was denaturated with 10% $HClO_4$ and the precipitated protein removed by centrifugation. The supernatant was neutralized with 400 μL of a mixture of 10 M KOH and 1 M potassium acetate and the potassium perchlorate precipitate removed by centrifugation. Unlabeled ADP (0.2 mM) was added to the supernatant and to the filtrate from micropartition before ion-exchange HPLC analysis. The peaks corresponding to ADP and ATP were collected and counted. For formyl phosphate analysis, 0.6 M neutral hydroxylamine was added to the retentate and filtrate before the addition of $HClO_4$ to the retentate. Unlabeled hydroxamate (1 mM) was added to the filtrate and the supernatant of the retentate after the $HClO_4$ treatment. The solutions were loaded on a 1-mL column of Dowex 1-Cl^- and eluted with H_2O to separate unreacted labeled formate from labeled hydroxamate. Each fraction was tested with the $FeCl_3$ reagent, and aliquots of the combined positive fractions were spotted on silica gel 60 TLC plates with H_2O-saturated butanol as the chromatographic solvent (Trams, 1967). The plates were sprayed with $FeCl_3$ reagent in 95% ethanol, and the colored spots were cut out and counted.

Positional Isotope Exchange Experiments. Positional isotope exchange (PIX) experiments were done at 37 °C in 10-mL volumes containing 100 mM triethanolamine buffer, pH 8.0, 8 mM [β,γ-^{18}O]ATP [prepared according to the method of Reynolds et al. (1983)], 40 mM sodium formate, 100 mM 2-mercaptoethanol, $MgCl_2$, and KCl as described and 10 μM enzyme active sites. When present, H_4folate and $PteGlu_3$ were 2 mM and 1 mM, respectively. Incubation was for 8–10 h.

Upon completion of the reaction, samples were quickly frozen and stored for later analysis. The reaction mixture was then diluted with 200 mL of H_2O, the pH was adjusted to 8.0, and the contents were loaded onto a DEAE-Sephadex A-25 column in the HCO_3^- form. The column was developed with an 800-mL linear (0.07–0.7 M) gradient of triethylamine (TEA)–HCO_3^-. Fractions (30 mL) were collected and monitored at 259 nm. Fractions containing the ATP were collected, pooled, and concentrated in vacuo. The white powder was repeatedly dissolved in absolute methanol and dried in vacuo to remove residual TEA–HCO_3^-. The product was then dissolved in 3 mL of absolute methanol and converted to the Na^+ salt by precipitation with 1 M NaI/acetone. The white pellet was then collected by centrifugation and resuspended in absolute acetone, and the centrifugation and suspension procedures were repeated. The product was dried in vacuo.

To prepare samples for NMR the product was dissolved in 0.6 mL of Hepes buffer, pH 8.0, further diluted with 10 mL of H_2O, and passed twice through a Chelex-100 cation-exchange resin. All glassware was rendered metal free by soaking in 50% H_2So_4–HNO_3 (v/v) followed by rinsing with distilled, deionized H_2O. Once passed through Chelex, samples were lyophilized and reconstituted with 0.6 mL of 33% HOD (Aldrich, low paramagnetic, T_1 = 48.1 s).

NMR spectra were obtained on a GE Nicolet 500 NMR interfaced with a Nicolet 1280 software package. Relative peak areas were obtained with the GEMCAP curve analysis deconvolution routine contained in the software package. NMR spectra were generated at 202.5 MHz (11.6 T) with a sweep width of ±500 Hz and spectral size of 16 384 data points. A 90° pulse width was used with an acquisition time of 8.19 s and a delay of 1 s. The T_1 of the β-phosphoryl group of the bridged species was 0.71 s. Typically, 1000 scans were necessary to generate a S/N of 200 and 95 for the experimental and control samples, respectively. Spectra were resolved to 1.1%.

The amount of ^{18}O-bridge-labeled ATP exchanged was determined by the method of Litwin and Wimmer (1979):

$$\text{exchange} = \frac{X}{\ln (1 - X)}[\text{ATP}]_0[\ln (1 - f)] \qquad (6)$$

where X if the fraction of ATP converted to product and $[ATP]_0$ is the original concentration of ATP. The term f is equal to $(P_t - P_0)/(P_\infty - P_0)$, where P_t, P_0, and P_∞ refer to the ^{18}O-non-bridge-labeled ATP present at time t, time zero, and equilibrium, respectively. Assuming torsional symmetry, the bridge label can scramble into any one of three positions. At time zero, enrichments consisted of 15.5% unlabeled, 10.7% nonbridge, and 73.7% bridge species.

Other Methods. Protein concentrations were determined by the method of Bradford (1976). (*R,S*)-H_4folate was synthesized and purified according to a published procedure (Samuel et al., 1970). Formyl phosphate was synthesized as described previously (Smithers et al., 1987) except that the temperature used in preparing the intermediate, formyl fluoride, was 90–95 °C.

Results

Evidence for Formate Kinase Activity Intrinsic to the Enzyme. In the absence of the third substrate, H_4folate, a slow but significant conversion of ATP and formate to ADP and formyl phosphate was observed in the presence of the enzyme. Formate-dependent ADP formation was shown to be linear with time, and the amount of product measured by the HPLC assay agreed well with the results from the coupled assay. Very little ADP formed in the absence of formate, ruling out possible ATPase contamination as an explanation for the results. The synthesis of formyl phosphate by the enzyme was demonstrated by chemically trapping the intermediate as formyl hydroxamate. The quantities of formyl phosphate formed in the presence of hydroxylamine corresponded to the amounts of ADP produced (data not shown).

Formate Kinase Activity Is Intrinsic to N^{10}-HCO-H_4folate Synthetase. The amount of enzyme required to detect the formate kinase reaction is approximately 10^4–10^5-fold higher than that used in the assay for the normal reaction. Because of this much lower rate, it was important to demonstrate that both activities are catalyzed at the same catalytic center and that the formate kinase activity is not caused by a contaminating enzyme. Three lines of evidence show that the activity is indeed due to the synthetase. First, coordinate loss of both normal and formate kinase activities occurred when the synthetase was irreversibly inactivated by heat (54 °C over a 50-min period) and a sulfhydryl reagent (2 mM *N*-ethylmaleimide over a 50-min period). Second, the formate kinase activity was lost when the tetramer was converted to the catalytically inactive monomer by removal of specific monovalent cations. Upon reassociation of the monomer to the tetramer by readdition of the monovalent cations, both the synthetase and formate kinase activities were regained at approximately the same rate (Figure 1). Third, the K_m values for ATP and formate in the kinase activity, 0.42 ± 0.05 mM and 2.2 ± 1.9 mM, respectively, were in good agreement with the corresponding K_m values in the synthetase reaction, 0.25 ± 0.13 mM and 8.3 ± 0.9 mM.

Characteristics of the Formate Kinase Activity. In order to compare the formate kinase activity with the normal synthetase activity, a variety of kinetic studies were done. The effects of divalent and monovalent cations on the rate of the formate kinase activity were examined. Stimulation of ADP formation was observed with increasing Mg^{2+} and K^+ concentrations. The K_m for both cations, 0.88 ± 0.15 mM for Mg^{2+} and 8.7 ± 2.6 mM for K^+ in the kinase activity, were comparable to those in the normal reaction, 2.3 mM for Mg^{2+} (Himes & Rabinowitz, 1962a) and 14 mM for K^+ (Himes & Wilder, 1965). An investigation of the effect of K^+ on the K_m of formate revealed that as the KCl concentration was increased from 0 to 50 mM the K_m of formate was lowered by about 6-fold from 8.3 ± 0.9 to 1.4 ± 0.9 mM. A similar effect was observed in the normal reaction wherein a 10-fold decrease in the K_m of formate was caused by NH_4^+ (Himes & Wilder, 1965).

Bicarbonate and acetate could substitute for formate but were less effective as substrates. The rate of ADP formation was 5-fold less with bicarbonate and 3-fold less with acetate compared to that with an equal concentration of formate. The K_m values of acetate (58 ± 13 mM) and bicarbonate (330 ± 14 mM) were much higher than the K_m of formate; however, the catalytic rate constants were about the same for the three substrates. Acetate- and bicarbonate-dependent ADP formation activities were also shown to be part of the catalytic center of the synthetase since no activity was retained when the tetramer was dissociated to the monomer.

Because formyl phosphate phosphorylation of ADP and formylation of H_4folate both require occupancy of the third substrate site, it was thought that a competitive inhibitor of H_4folate would stimulate the formate kinase activity of the enzyme. $PteGlu_3$, the oxidized form of the natural coenzyme in *Clostridia* (Rabinowitz & Himes, 1960) and one of the forms of naturally occurring coenzymes in eucaryotes

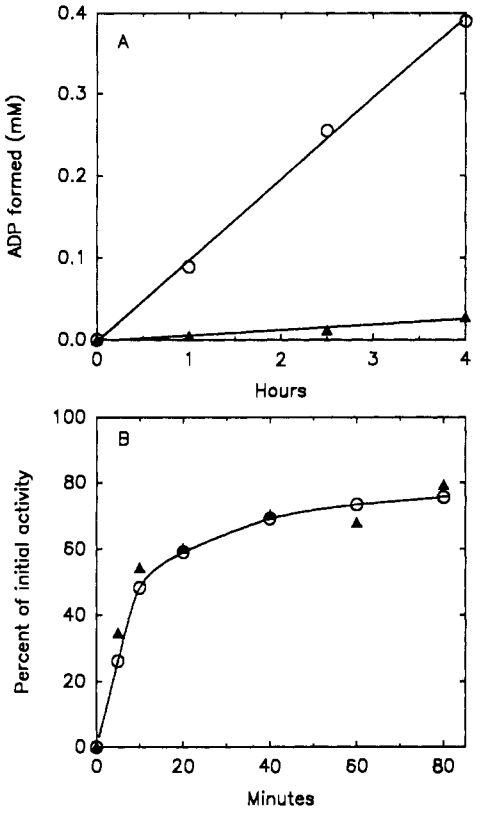

FIGURE 1: Formate kinase activity is associated with the tetramer form of the protein. (A) Inactivation of formate kinase activity by removal of monovalent cations to form the monomer. The cations were removed by exchanging the enzyme (1.2 mg/mL) into 25 mM Tris-HCl, pH 8, on a Sephadex G-25 column as described under Experimental Procedures. The formate kinase activity of the tetramer (○) and monomer (▲) was measured with 10 μM enzyme sites and the HPLC assay for ADP formation. (B) Coordinate reactivation of synthetase and formate kinase activities during cation-induced reassociation to the tetramer. The monomer (0.1 mg/mL) was reassociated to the tetramer in 25 mM Tris-HCl, pH 8, containing 100 mM NH_4Cl, 10 mM Na_2SO_4, and 50 mM 2-mercaptoethanol at 20 °C. Aliquots were withdrawn at different time periods and assayed for synthetase (○) and formate kinase (▲) activities.

(McGuire & Coward, 1984), was prepared by solid-phase peptide synthesis. This compound was found to be a competitive inhibitor of H_4folate with a K_i value of 0.82 ± 0.02 mM, close to the K_m of H_4folate (0.28 ± 0.12 mM). The effect of $PteGlu_3$ on the formate kinase reaction was slight, stimulating the reaction rate approximately 2-fold with the bacterial enzyme and 1.5-fold with the yeast enzyme.

Phosphonoacetaldehyde ($HCOCH_2PO_3^{2-}$), which is a nonhydrolyzable analogue of formyl phosphate, was tested for its inhibitory activity in the normal and formate kinase reactions as well as in the forward and reverse reactions with formyl phosphate as a substrate. It was found to be a noncompetitive inhibitor of formate and ATP in the formate kinase reaction and of formate in the normal reaction (Table I). However, when ATP and formate concentrations were varied at a constant ratio, competitive inhibition was observed in both reactions. Phosphonoacetaldehyde was also a competitive inhibitor of formyl phosphate in the forward and reverse formyl phosphate supported reactions. The inhibition constants for phosphonoacetaldehyde in all four reactions were similar (Table I).

Kinetic Mechanism of the Formate Kinase Activity. The kinetic mechanism of the formate kinase reaction was determined from initial velocity and product inhibition studies. When ATP or formate concentrations were varied at five fixed levels of the other substrate, ranging from a nonsaturating to a saturating concentration, the lines of the reciprocal plots intersected in the upper-left quadrants, indicating a sequential mechanism. Slope replots of the primary data intersected the y axis at a positive value. ADP was found to be a noncompetitive inhibitor against ATP and formate at both saturating and unsaturating levels of the second substrate. These results taken together appear to rule out rapid equilibrium random and rapid equilibrium ordered mechanisms but are consistent with a sequential random bi bi mechanism for the formate kinase reaction.

Table I: Inhibition of Several Enzyme Activities by Phosphonoacetaldehyde

reaction	variable substrate	pattern[a]	K_i (mM)
formate kinase	MgATP	NC	1.93 ± 0.11
	formate	NC	3.78 ± 0.07
	MgATP and formate (constant ratio)	C	2.31 ± 0.05
normal (synthetase)	formate	NC	2.84 ± 0.03
	MgATP and formate (constant ratio)	C	5.41 ± 0.89
formyl phosphate supported (forward)	formyl phosphate	C	7.44 ± 0.24
formyl phosphate supported (reverse)	formyl phosphate	C	1.23 ± 0.15

[a] NC = noncompetitive; C = competitive.

Exchange of ADP into ATP. Considering the suggested mechanism of the reaction, involving the formation of enzyme-bound formyl phosphate, it might be expected that the enzyme would catalyze a formate-dependent ADP ↔ ATP exchange. Previous work (Himes & Rabinowitz, 1962b) failed to detect such an activity. Because in those studies a significant exchange occurred in the absence of formate and the enzyme used in the current work has been through several more purification steps, this experiment was repeated. With use of up to 60 μM bacterial enzyme sites, a very slow exchange of ADP into ATP occurred at 37 °C. After 200 min, only 6% of the theoretical amount of exchange had occurred. This rate was stimulated approximately 30% by the inclusion of formate.

Burst Kinetics of the Formate Kinase Reaction. To explain the slow utilization of synthetic formyl phosphate in the reaction, it has been proposed that there is a kinetic barrier to the binding and release of the putative intermediate (Smithers et al., 1987). A slow release of ADP and formyl phosphate in the formate kinase reaction could explain the slow rate of this activity. If this is the case, there should be a burst of product formation on the enzyme. To detect a possible burst reaction, ADP produced was measured after short periods of time by denaturing the protein before ADP analysis. Results of such an experiment are shown in Figure 2A. It is apparent that the lines representing the rate of ADP formation intersect the ordinate at ADP concentrations roughly equal to the amounts of enzyme present. This is consistent with the formation of a burst of enzyme-bound ADP.

For analysis of the burst of product formation in the formate kinase reaction at much faster reaction times (as low as 100 ms), a multimixing apparatus was used. The results of this rapid-quench experiment are presented in Figure 2B. The time course is characterized by a transient burst phase in the pre-steady-state and a steady-state phase. The steady-state rate constant k_{ss} was obtained from the slope of the line and was estimated to be 0.024 ± 0.006 s^{-1}. The transient burst height, which is determined by extrapolation of the line representing the steady-state rate to zero time, is equivalent to about 80% of the total enzyme concentration. The transient rate constant (λ) was evaluated from the slope of a first-order

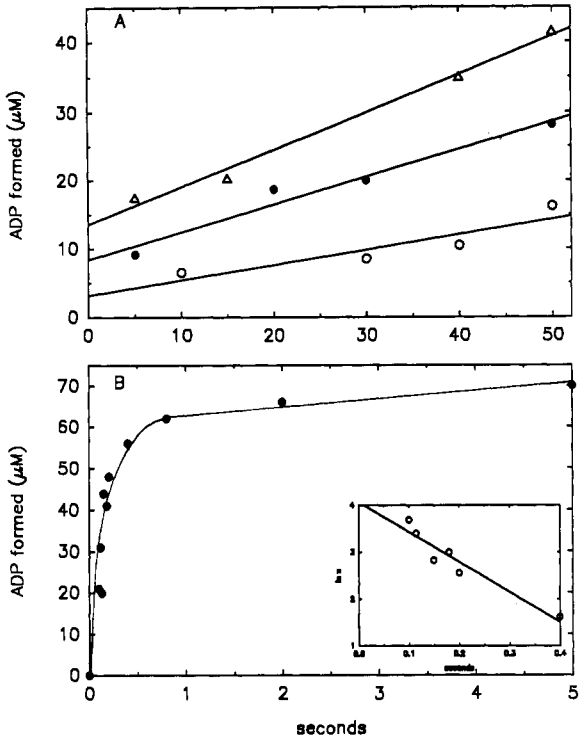

FIGURE 2: Demonstration of a burst of ADP formation on the enzyme in the formate kinase reaction. (A) Enzyme at active site concentrations of 5 (○), 10 (●), and 15 (△) μM was incubated at 37 °C with 5 mM ATP andd 40 mM formate. Aliquots were taken at the times shown and diluted 3-fold into 0.5 M $NH_4H_2PO_4$, pH 4.3, before HPLC analysis for ADP. (B) The enzyme was rapidly combined with formate and [^{3}H]ATP so that the final concentrations were 75 μM, 40 mM, and 5 mM, respectively. The reaction was quenched with 0.36 M HCl in a Durrum multimixing apparatus. The amount of [^{3}H]ADP formed was measured by HPLC. The inset is a first-order plot of the reaction during the burst phase.

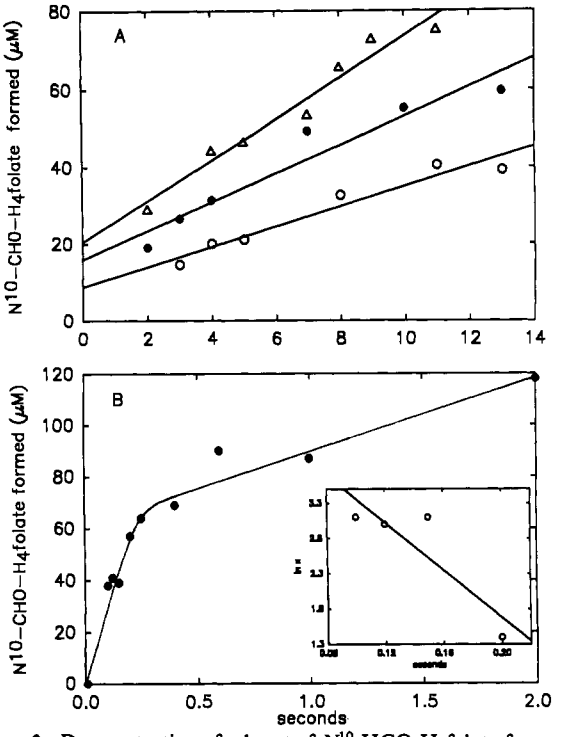

FIGURE 3: Demonstration of a burst of N^{10}-HCO-H_4folate formation from a reaction between H_4folate and E–ADP–formyl phosphate complex. (A) H_4folate (2 mM) was added to a reaction mixture containing a final concentration of 10 (○), 20 (●), and 25 (△) μM. The experiment was done at 5 °C. The reaction was manually quenched with 0.36 M HCl at different time intervals, and the absorbance at 350 nm was measured. (B) Time course of the burst at reaction times shorter than 2 s with a multimixing apparatus. The reaction conditions were the same as in (A) except the final enzyme site concentration was 75 μM. The inset is similar to that in Figure 2B.

Table II: Demonstration of Enzyme-Bound ADP and Formyl Phosphate by Micropartition[a]

enzyme sites (μM)	product measured	retentate (μM)	filtrate (μM)
250	ADP	271	71
70	ADP	68	5
60	formyl phosphate	51	17

[a] The assay mixture containing [^{3}H]ATP or [^{3}H]formate was incubated for 10 s at 37 °C as described under Experimental Procedures. After being cooled in ice, the solution was centrifuged through the micropartition apparatus, and the retentate and filtrate were analyzed for the products as described.

plot (inset in Figure 2B) of the logarithm of the difference (x) between the burst amplitude (β) and the amount of product formed at different times during the burst phase. The burst rate constant of the pre-steady-state phase was calculated to be 6.4 ± 1.0 s^{-1}.

The formation of enzyme-bound ADP and formyl phosphate was further demonstrated by the micropartition assay. The results of this assay, which separates enzyme-bound ligands from free ligands, are summarized in Table II. If ADP and formyl phosphate synthesized by the enzyme were free, the concentrations of product in the filtrate and retentate would have been equal. The much higher concentration of these products in the retentate compared to that in the filtrate indicated that most of the ADP and formyl phosphate were still bound to the enzyme.

Burst Kinetics in the Formylation of H_4folate by Formyl Phosphate. In a previous paper (Smithers et al., 1987), it was demonstrated that formyl phosphate could formylate H_4folate in the presence of enzyme and ADP. By using higher protein concentrations than those used earlier and a multimixing apparatus, we now show that this reaction proceeds with burst kinetics. The reaction of H_4folate with the E–ADP–formyl phosphate complex was done at 5 °C because the burst rate was too fast to be measured at 37 °C with the instrumentation available to us. The time course for the formation of N^{10}-HCO-H_4folate at 5 °C is presented in Figure 3A. Extrapolation of the data to zero time shows that a burst of product formation, approximately equal to the concentration of enzyme sites, occurred. This transient pre-steady-state burst can be detected at shorter times as shown in Figure 3B. The burst height (β) in this experiment corresponded to 80% of the enzyme concentration used. The steady-state rate constant was calculated to be 0.40 ± 0.07, which is in reasonable agreement with the average rate constant, 0.22 ± 0.02, obtained in Figure 3A. The transient rate constant, 18 ± 6 s^{-1}, was about equal to the catalytic rate constant of the overall reaction (14 ± 1 s^{-1}) determined under similar conditions. The UV–vis spectrum of the product after 100 ms and after quenching with HCl was that of N^5,N^{10}-methenylH_4folate.

Positional Isotope Exchange Experiments. The PIX experiments were done under several conditions: in the presence of all three substrates (ATP, formate, and H_4folate), of ATP and formate, of ATP, formate, and $PteGlu_3$, and of ATP alone. Depending on the components present, 60%–90% of the initial ATP remained at the end of the incubation. Evidence for PIX was not obtained under any set of conditions. The NMR spectrum of the β-phosphoryl group of the starting [β,γ-^{18}O]ATP showed three sets of triplets, two of which are shifted upfield by the isotope effect imparted by ^{18}O in the bridge and nonbridge positions. The isotope shifts amounted to 0.16 ppm

Table III: Comparison of Rate Constants for Procaryotic and Eucaryotic Enzymes

reaction	k_{cat} (s^{-1}) bacterial enzyme	yeast enzyme
normal	278 ± 51[a]	200 ± 47[b]
formyl phosphate supported (forward)	5.1 ± 0.9[c]	0.47 ± 0.10[d]
formyl phosphate supported (reverse)	0.16 ± 0.04[c]	0.021 ± 0.012[d]
formate kinase[e]	0.024 ± 0.006	$5.7 \times 10^{-5} \pm 1.0 \times 10^{-6}$

[a] T = 37 °C; pH 8.0. Assay conditions are described under Experimental Procedures. [b] T = 37 °C; pH 7.5. Assay conditions are described under Experimental Procedures. [c] T = 20 °C; pH 7.0. Data taken from Smithers et al. (1987). [d] T = 20 °C; pH 7.0. Assay conditions are described under Experimental Procedures. [e] T = 37 °C; pH 8.0. Assay conditions are described under Experimental Procedures.

for the ^{18}O bridge species and 0.27 ppm for the ^{18}O nonbridge species. This is in agreement with the isotope shifts reported originally by Cohn and Hu (1980). Curve deconvolution analysis using the GEMCAP program yielded no significant difference between the spectra of the starting [^{18}O]ATP and that isolated from various experiments, within the limits one can reliably detect with respect to the signal:noise ratio.

Catalytic Mechanism of the Eucaryotic Enzyme. C_1-H_4-folate synthase, which contains N^{10}-HCO-H_4folate synthetase, catalyzes a formate kinase activity but at a rate of 100-fold slower than that of the bacterial enzyme. In addition, chemically synthesized formyl phosphate was demonstrated to support the formylation of H_4folate in the forward direction and phosphorylation of ADP in the backward direction with the yeast enzyme. A comparison of the rate constants for these partial reactions with those of the bacterial and yeast enzymes is presented in Table III.

Discussion

Although previous studies have provided convincing evidence for the involvement of formyl phosphate as a nondissociable intermediate in the catalytic mechanism of N^{10}-HCO-H_4folate synthetase, none of them demonstrated the actual formation of the intermediate by the enzyme. In this study, a slow but kinetically significant synthesis of formyl phosphate from formate and ATP in the absence of H_4folate was demonstrated. Several lines of evidence indicate that this formate kinase activity is intrinsic to the synthetase. Among them were a similar monovalent cation dependence for the synthetase and formate kinase activities, the lack of formate kinase and synthetase activities in the monomer, the simultaneous increase in both activities during reassociation of the monomer to the tetramer, the lowering of the K_m of formate with increasing levels of K^+ in both reactions, similar K_m values for ATP, formate, and Mg^{2+} and K_i values for phosphonoacetaldehyde, and the fact that coordinate inactivation of both activities by heat and a sulfhydryl reagent occurred. The failure to detect the formate kinase activity in earlier studies is probably due to the facts that much lower enzyme concentrations were used in the earlier works and that a significant ATPase activity was present in the earlier preparations of the enzyme.

The slow rate of formyl phosphate synthesis can be explained by the slow dissociation of the products from the enzyme as proposed earlier (Smithers et al., 1987). Supporting evidence for this came from the observed burst of ADP formation (Figure 2) and from the demonstration that ADP and formyl phosphate remain bound to the enzyme with the micropartition method (Table II). From rapid-quench studies in which ATP and formate were rapidly combined with the enzyme, the rate constant of this burst was found to be 270 times faster than the steady-state rate of the formate kinase activity. The lack of a significant formate-dependent exchange between ADP and ATP is also consistent with a slow dissociation of ADP from the enzyme. The simplest scheme for the mechanism of the formate kinase reaction based on our findings is illustrated in eq 7. The rate constants for the reaction scheme

$$E + ATP + HCOO^- \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} [E\text{–}ATP\text{–}HCOO^- \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} E\text{–}ADP\text{–}formyl\ phosphate] \xrightarrow{k_3} E + ADP + formyl\ phosphate \quad (7)$$

in eq 7 were calculated from the rapid-quench data with the equations described by Shafer et al. (1972). Using the experimental values for k_{cat} = 0.024 ± 0.006 s^{-1}, β/E = 0.81 ± 0.02, and λ = 6.39 ± 0.96 s^{-1}, the rate constants were determined to be k_2 = 5.21 ± 0.47 s^{-1}, k_{-2} = 1.16 ± 0.06 s^{-1}, and k_3 = 0.0295 ± 0.0003 s^{-1}. Thus the product release step, k_3, is rate determining for the formate kinase reaction.

Although the formation of formyl phosphate in the enzyme complex occurs at a rate much faster than the steady-state rate, the rate constant is still about 50–100 times lower than the overall catalytic rate constant. Thus, it does not in itself provide conclusive evidence that formyl phosphate is an intermediate in the normal reaction pathway. However, that formyl phosphate could serve as a kinetically competent intermediate was demonstrated in the formylation reaction of H_4folate by formyl phosphate in the presence of ADP. This reaction is also characterized by a burst phase followed by a slower steady-state phase. The burst experiments were done at a much lower temperature (5 °C) to decrease the rate of the formylation reaction and to allow it to be measured by our rapid-quench instrument. The rate constant for the burst reaction at 5 °C (18 s^{-1}) is considerably larger than that for the steady-state phase (0.40 s^{-1}), and it is approximately equal to the rate constant of the overall enzymatic reaction at the same temperature (14 s^{-1}).

The mechanistic and kinetic data we obtained are summarized in a proposed scheme for the overall sequential reaction showing formyl phosphate as the nondissociable intermediate (Figure 4). The formate kinase activity with a very slow product release step is depicted as a side pathway (left side). The overall normal reaction is shown in the middle box. As proposed earlier (Smithers et al., 1987), a kinetic barrier to the release of the intermediate may be as slow enzyme isomerization process (E to E*), which converts the E–MgADP–H_4folate–formyl phosphate to a form from which formyl phosphate can dissociate (right side).

The formate kinase reaction was much slower than the reactions which utilize formyl phosphate in the forward and reverse reactions (Table III). The latter reactions are limited by the binding of formyl phosphate rather than its release, which possibly could explain this difference. But another factor is the fact that in the formyl phosphate utilizing reactions all three substrate binding sites were occupied, which undoubtedly exerts a major influence on the efficiency of the catalytic center. To simulate this situation, we included $PteGlu_3$, an inactive analogue of H_4folate, in the assay mixture. The stimulation of ADP formation by $PteGlu_3$, however, was quite weak, only 2-fold with the bacterial enzyme and 1.5-fold with the yeast enzyme. The lack of a large enhancement effect on the catalytic rate may be due to the shortcomings of $PteGlu_3$ as an analogue of H_4folate. Even if $PteGlu_3$ is a good competitive inhibitor of the actual substrate, there are some important structure differences between $PteGlu_3$ and the reduced

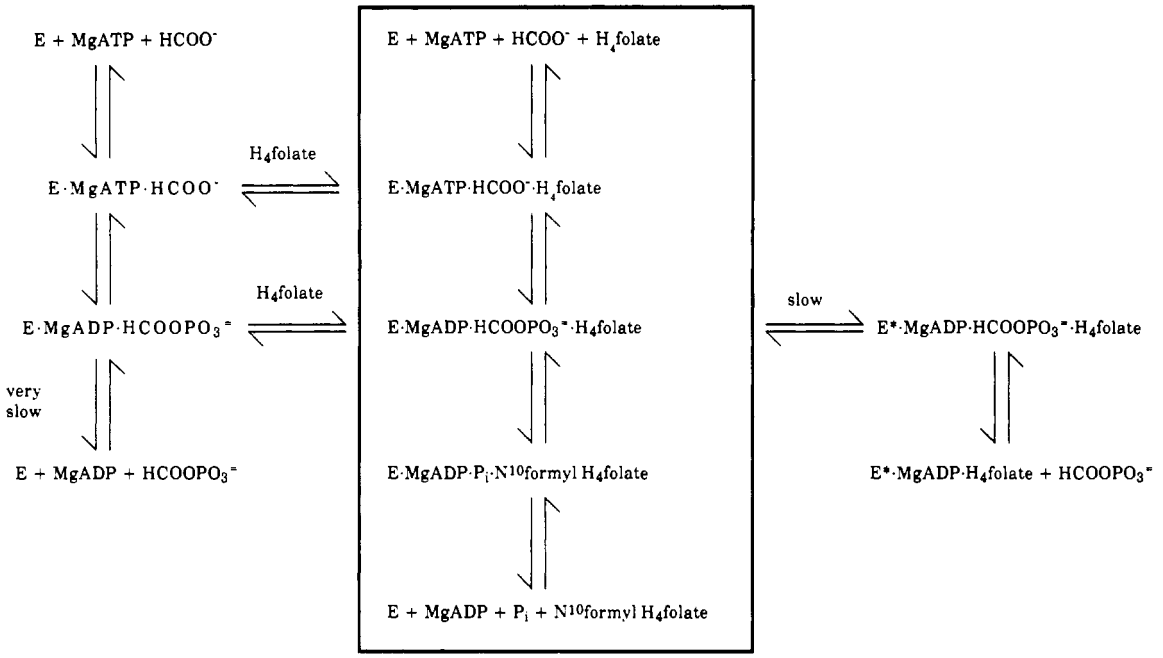

FIGURE 4: Scheme showing the catalytic mechanism for the reaction catalyzed by N^{10}-HCO-H_4folate synthetase. The formation of formyl phosphate on the enzyme is depicted in the formate kinase reaction (left) and in the normal reaction (middle).

substrate. The pteridine ring in $PteGlu_3$ is planar, and the substituents of the oxidized and reduced forms of the ring have different pK_a values (Temple & Montgomery, 1984). It is conceivable that a reduced pteridine ring is required to produce the proper stereochemistry and essential types of interactions of the folate substrate with the enzyme's functional group in order to fully mimic the conformational role of H_4folate. Indeed, evidence has been presented which indicates that the reduced form of $PteG_3$, but not the oxidized form, causes a conformational change in the eucaryotic enzyme (Strong et al., 1987).

The lack of positive results from the PIX experiments, while not providing additional evidence to support formyl phosphate as an intermediate, does not argue against the proposed mechanism. The lack of exchange when H_4folate was present can be explained by assuming that formyl transfer from the putative intermediate to H_4folate is fast compared to phosphorylation of ADP. Hence, little opportunity is afforded for the reverse reaction of the formate kinase step to occur. The lack of exchange in the absence of H_4folate with or without $PteGlu_3$ present, conditions under which the formate kinase reaction occurred, is not so readily interpretable. Since the k_{off} of the putative formyl phosphate is projected to be very slow, ample time should be available for the reverse reaction to occur on the enzyme. Clearly, our results show this not to be the case. From the limits of detection by NMR, we have determined the upper limit of exchange to turnover that could go undetected by this method and have found it to be <0.01 exchange:turnover.

One possible explanation for the negative PIX results during the formate kinase reaction is that the reverse reaction is not energetically favorable. However, the kinetic studies of the burst phase demonstrated that this is not the case; k_{-2} was about 20% of k_2. Another explanation is that the k_{off} of ATP is very low. Exchange of free ATP with enzyme-bound ATP is necessary to detect PIX which occurs in the complex. A third explanation for the lack of PIX is restricted rotation of the β-phosphoryl group of ADP. Although the rotational barrier about the P–O bond has been estimated to be low in the case when no enzyme is present ($\Delta G^{\#} \approx 1$ kcal, $k_{rot} = 10^{12}$ s^{-1}) (Engelke, 1973), the situation at the enzyme's active site may not permit much torsional freedom. Interaction of both the nucleophile and leaving group with the divalent metal ion, as recently shown by Herschlag and Jencks (1987), may impose restrictions to the torsional symmetry about the O–P_β bond. Biotin carboxylase also shows a lack of PIX (Tipton & Cleland, 1988b; Ogita & Knowles, 1988), and it has been suggested that the complexation of ATP by divalent metal ions at the active center of biotin carboxylase restricts the torsional freedom of the β-phosphoryl group (Tipton & Cleland, 1988a). Some ATP-utilizing enzymes in addition to N^{10}-CHO-H_4folate synthetase and biotin carboxylase which do not demonstrate PIX at the $P_\beta O$–P_γ bond cleavage are nitrogenase (Matsunaga, 1987), succinyl-CoA synthetase (Williams & Bridger, 1987), and myosin (Dale & Hackney, 1987). Several examples which show PIX are glutamine synthetase (Midelfort & Rose, 1976; Wimmer et al., 1979; Villafranca & Raushel, 1980), carbamoylphosphate synthetase (Balakrishnan, 1978; Raushel & Villafranca, 1980; Rubio et al., 1981; Meek et al., 1987), and CTP synthetase (von der Saal et al., 1985).

Since the yeast C_1-H_4folate synthase also catalyzes a formate kinase activity and formyl phosphate supports the forward and reverse synthetase reactions in the eucaryotic enzyme, it can be concluded that the overall reaction also proceeds by a catalytic mechanism utilizing formyl phosphate as an intermediate. The differences in the rates of the normal, formyl phosphate supported and formate kinase reactions catalyzed by the two enzymes (Table III) are probably explained by structural differences between the bacterial and yeast enzymes leading to different types of interactions between the substrates and the enzyme's functional groups.

The formate kinase activity of N^{10}-HCO-H_4folate synthetase is analogous to activities catalyzed by glutamine synthetase (Krishnaswamy et al., 1962), glutamyl-γ-cysteine synthetase (Meister, 1974), and glutathionine synthetase (Meister, 1974). All these enzymes catalyze three-substrate reactions involving the synthesis of an amide bond from an amine and a carboxylate substrate accompanied by the cleavage of the γ-phosphate–oxygen bond of ATP. In the absence of the amine, a carboxylate-stimulated formation of ADP from ATP occurs. As in the formate kinase activity of N^{10}-HCO-H_4folate synthetase, there is considerable evidence that acyl phosphates

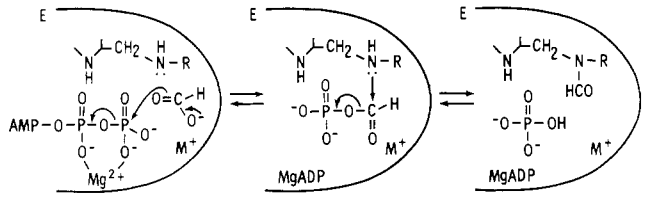

FIGURE 5: Proposed catalytic mechanism of N^{10}-H_4folate synthetase utilizing formyl phosphate as an intermediate.

are formed along with ADP in these partial reactions. These synthetases do not catalyze exchange between ADP and ATP in the absence of the amine substrate. In addition, enzymes that catalyze carboxylation reactions such as carbamoyl-phosphate synthetase (Anderson & Meister, 1966) and biotin carboxylase (Climent & Rubio, 1986) also catalyze a bicarbonate-dependent conversion of ATP to ADP, suggesting the formation of carboxy phosphate intermediates. From continued studies of such enzymes, a similar catalytic center for the carboxylate activation part of these reactions may emerge.

The suggested mechanism of the reaction catalyzed by N^{10}-HCO-H_4folate synthetase is presented in Figure 5. As illustrated, a sequential mechanism is operative wherein enzyme-bound phosphate is initially formed from ATP and formate, followed by another nucleophilic reaction between the formyl group of formyl phosphate and the 10-nitrogen of H_4folate. Results to date do not rule out an initial phosphorylation of an enzyme functional group by ATP. Evidence that a slow formation of formyl phosphate does occur in the absence of H_4folate and that the formyl phosphate is an active substrate in the forward and reverse reactions is consistent with this mechanism. Another important point emphasized in this model is that all three substrates must be present to produce a catalytically efficient structure at the active site. This is strongly supported by kinetic (Joyce & Himes, 1966a,b; McGuire & Rabinowitz, 1978) and NMR and EPR data (Buttlaire et al., 1975a,b). Distance measurements from NMR data (Wendland et al., 1983; Yeh et al., 1988) place the monovalent cation near the MnADP and formate binding sites. The cation may be important in the binding of formate, since its presence causes a large decrease in the K_m of formate.

ACKNOWLEDGMENTS

We thank Dr. Michael Doughty for his help in the synthesis of $PteGlu_3$.

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Bacterial Sarcosine Oxidase: Identification of Novel Substrates and a Biradical Reaction Intermediate†

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Received December 21, 1988; Revised Manuscript Received March 15, 1989

ABSTRACT: Corynebacterial sarcosine oxidase contains both covalently and noncovalently bound FAD and forms complexes with various heterocyclic carboxylic acids (D-proline and 2-furoic, 2-pyrrolecarboxylic, and 2-thiophenecarboxylic acids). 2-Furoic acid, a competitive inhibitor with respect to sarcosine, selectively perturbs the absorption spectrum of the noncovalent flavin, suggesting that the enzyme has a single sarcosine binding site near the noncovalent flavin. Several heterocyclic amines have been identified as new substrates for the enzyme. Similar reactivity is observed with L-proline and L-pipecolic acid whereas L-2-azetidinecarboxylic acid is less reactive. Turnover with L-proline is slow (TN = 4.4 min^{-1}) as compared with sarcosine (TN = 1000 min^{-1}). Anaerobic reduction of the enzyme with heterocyclic amine substrates at pH 8.0 occurs as a biphasic reaction. A similar long-wavelength intermediate is formed in the initial fast phase of each reaction and then decays in a slower second phase to yield 1,5-dihydroFAD. The slow phase is not kinetically significant during aerobic turnover at pH 8.0 and is absent when the anaerobic reactions are conducted at pH 7.0. EPR and other studies at pH 7.0 show that the long-wavelength species is a half-reduced form of the enzyme (1 electron/substrate-reducible flavin) containing 0.9 mol of flavin radical/mol of substrate-reducible flavin. This biradical intermediate exhibits an absorption spectrum similar to that expected for a 50:50 mixture of red anionic and blue neutral flavin radicals. A similar long-wavelength species is observed during titration of the enzyme with sarcosine and other reductants. Studies with L-proline suggest that reduction of the enzyme involves initial transfer of two electrons to the noncovalent flavin. The covalent flavin is not required and can be complexed with sulfite without affecting the rate of electron transfer. The initial half-reduced form of the enzyme appears to be rapidly converted to the biradical form via comproportionation of the reduced noncovalent flavin with the oxidized covalent flavin.

Sarcosine oxidase from *Corynebacterium* sp. P-1 is an inducible enzyme that is synthesized in large amounts (3% of total protein) when the organism is grown with sarcosine as the source of carbon and nitrogen (Kvalnes-Krick & Jorns, 1986). The enzyme catalyzes the oxidative demethylation of sarcosine to yield formaldehyde, glycine, and hydrogen peroxide. The enzyme binds tetrahydrofolate (2 mol/mol of enzyme). The presence of tetrahydrofolate does not affect the rate of sarcosine oxidation, but 5,10-methylenetetrahydrofolate is formed as a reaction product in place of formaldehyde (Kvalnes-Krick & Jorns, 1987). Sarcosine oxidase contains both covalently bound flavin adenine dinucleotide (FAD)[1]

† This work was supported in part by Grants GM 31704 (M.S.J.) and AR 38917 (R.H.) from the National Institutes of Health.
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0006-2960/89/0428-5145$01.50/0