Mechanism for the Coupling of ATP Hydrolysis to the Conversion of 5-Formyltetrahydrofolate to 5,10-Methylenetetrahydrofolate*

(Received for publication, April 17, 1995)

Teng Huang and Verne Schirch†

From the Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298

5,10-Methylenetetrahydrofolate synthetase catalyzes the irreversible conversion of 5-formyl-tetrahydropteroylpolyglutamates (5-CHO-H₄PteGluₙ) to 5,10-methylenetetrahydropteroylpolyglutamates (5, 10-CH–H₄PteGluₙ). The equilibrium of the nonenzymatic reaction, which equilibrates slowly in the absence of enzyme, greatly favors 5-CHO-H₄PteGluₙ. The enzyme couples the reaction to the hydrolysis of ATP shifting the equilibrium to favor 5,10-CH–H₄PteGluₙ. Substrate-dependent non-equilibrium isotope exchange of [³H]ADP into ATP was observed, suggesting the formation of a phosphorylated intermediate of 5-CHO-H₄PteGluₙ during the enzyme-catalyzed reaction. The competitive inhibitor 5-formyltetrahydrohomofolate also supported the ADP to ATP exchange, suggesting that this molecule could also form a phosphorylated intermediate. The initial rates of the ADP-ATP exchange with saturating ADP were about 70 s⁻¹ for both compounds, while the kₐ values for product formation were 5 s⁻¹ for 5-CHO-H₄PteGluₙ, and 0.005 s⁻¹ for 5-formyltetrahydrohomofolate. Starting with 5-[³¹]OCHO-H₄PteGluₙ, it was shown by ³¹P NMR that the formyl oxygen of the substrate was transferred to the product phosphate during the reaction. This further supports the existence of a phosphorylated intermediate. The formyl group of 5-CHO-H₄PteGluₙ is known to be an equilibrium mixture of two rotamers. Stop-flow analysis of the enzymatic reaction showed that only one of the rotamers serves as a substrate for the enzyme.

5,10-Methylenetetrahydrofolate synthetase (MS)¹ (EC 6.3.3.2), also referred to as 5-formyltetrahydrofolate cyclohydrolase, catalyzes the ATP-dependent conversion of 5-formyltetrahydrofolate (5-CHO-H₄PteGluₙ) to 5,10-methylenetetrahydrofolate (5,10-CH–H₄PteGluₙ) (Reaction I). The enzyme activity was first found in sheep liver acetone powder extracts by Peters and Greenberg (1) and later studied by Kay et al. (2, 3). This enzyme has been purified to homogeneity from both prokaryotic and eukaryotic sources (4-6). The primary structure of the enzyme from rabbit liver has been determined (7).

5-CHO-H₄PteGluₙ + ATP → 5,10-CH–H₄PteGluₙ + ADP + P₃

**Reaction I**

The origin and function of 5-CHO-H₄PteGluₙ has been a source of confusion and debate (8). The acid-catalyzed interconversion of 5-CHO-H₄PteGluₙ and 5,10-CH–H₄PteGluₙ occurs nonenzymatically with the equilibrium greatly favoring 5-CHO-H₄PteGluₙ at pH 7 (2, 9). The rate of the nonenzymatic reaction occurs on the time scale of hours under physiological conditions and is complicated by 5,10-CH–H₄PteGluₙ also being hydrolyzed to 10-CHO-H₄PteGluₙ. By coupling Reaction I to ATP hydrolysis, the equilibrium is shifted to favor 5,10-CH–H₄PteGluₙ at pH 7. For many years, neither an enzymatic source nor function of 5-CHO-H₄PteGluₙ, was known. This suggested that the function of MS was to serve as a salvage pathway for the reincorporation of this nonenzymatically produced folate derivative into the one-carbon pool.

5-CHO-H₄PteGluₙ has been found to occur in many cells at low concentrations (8). However, its presence in cells is suspect since the harsh procedures used to extract folates are known to convert 10-CHO-H₄PteGluₙ to 5-CHO-H₄PteGluₙ. Recently, it was shown that in Neurospora crassa conidiospores at least 85% of the folate pool is 5-CHO-H₄PteGluₙ (10). The mild extraction procedure was shown not to account for the presence of the 5-CHO-H₄PteGluₙ. Because 5-CHO-H₄PteGluₙ is the only tetrahydrofolate derivative that is stable to oxidative degradation, these studies suggested that a physiological role is to serve as a storage form of this enzyme in dormant stages of cellular life cycles. It has also been noted that 5-CHO-H₄PteGluₙ is an inhibitor of many enzymes in one-carbon metabolism, suggesting it may also serve a regulation role (8). Making Reaction I irreversible would be beneficial to a cell whether 5-CHO-H₄PteGluₙ is formed nonenzymatically, is used as a storage form for folates, or functions in regulation by inhibiting enzymes in one-carbon metabolism.

5-CHO-H₄PteGluₙ, clinically known as leucovorin, has been administered to cancer patients to rescue them from toxicity during high dose methotrexate chemotherapy, or more recently to enhance the cytotoxic activity of 5-fluorouracil (11, 12). MS is the only known enzyme that utilizes 5-CHO-H₄PteGluₙ as a substrate. For 5-CHO-H₄PteGluₙ to be effective as a rescue agent and an enhancer of 5-fluorouracil toxicity, it must first be converted to 5,10-CH–H₄PteGluₙ through MS activity. The effectiveness of leucovorin in chemotherapy is at least partially dependent on the irreversibility of Reaction I. It has been shown that doubling intracellular concentrations of 5-CHO-H₄PteGluₙ by inhibiting MS activity, suppressed growth of MCF-7 human breast cells by 80% (13). This suggests that MS could be a potentially important enzyme as a target in chemotherapy.

---

¹ This work was supported by Grant GM 28143 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 804-828-9482; Fax: 804-828-1473.

¹ The abbreviations used are: MS, 5,10-methylenetetrahydrofolate synthetase; 5-CHO-H₄PteGluₙ, 5-formyltetrahydropteroylglutamate containing n number of glutamate residues; 5,10-CH–H₄PteGluₙ, 5,10-methylenetetrahydropteroylglutamate; 10-CHO-H₄PteGluₙ, 10-formyltetrahydropteroylglutamate; 5-CHO-tetrahydrofolate, 5-formyltetrahydrofolate; Kₘ, the rate constant for the exchange rate of [¹⁵C]ADP into ATP.
In this paper, we report the mechanism of the coupling of ATP hydrolysis to the conversion of 5-CHO-H\textsubscript{4}PteGlu to 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu by MS. By using NMR spectroscopy and isotope exchange techniques, a 5-CHO-H\textsubscript{4}PteGlu, phosphorylated intermediate was demonstrated. The role of this intermediate in making Reaction I irreversible is discussed.

**EXPERIMENTAL PROCEDURES**

Materials—(6R,6S)-5-CHO-H\textsubscript{4}PteGlu (folinic acid, calcium salt), MgATP, ADP (sodium salt), 1 mM MgCl\textsubscript{2}, standard solution, and other buffer reagents were purchased from Sigma and used without further purification. [2,8-\textsuperscript{18O}]ADP (15 Ci/mmol) was purchased from Moravek Biochemicals Inc. H\textsubscript{2}\[18O\] (95% enriched) was purchased from ICON Services, Inc. 6S)-5-CHO-H\textsubscript{4}PteGlu or 28\textsuperscript{16O}-labeled 5-CHO-tetrahydrohomofolate was kindly provided by Dr. Barry Shane (University of California at Berkeley) and further purified by HPLC on a Spherisorb C\textsubscript{18} column. The compound was eluted with a gradient from 50 mM KMES, pH 6.0, to 10% acetonitrile in H\textsubscript{2}O, and the concentration was determined by using the molar absorption coefficient of 5-formyltetrahydrofolate at 287 nm (\(\varepsilon = 31,500\) M\(^{-1}\) cm\(^{-1}\)) (14). Rabbit livers were from Pel-Freeze Biologicals (Rogers, AK). Livers were quick-frozen and shipped within 24 h on dry ice after removal from the rabbits and stored at \(-70\) °C. MS was purified from the liver homogenate by using a previously described procedure (14) determined by using the A\textsubscript{280} extinction coefficient of 15 \(\mu\)M \(^{-1}\) cm\(^{-1}\). 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu. Preparation—Either 15 mg of (6R,6S)-5-CHO-H\textsubscript{4}PteGlu or 5 mg of (6S)-5-CHO-H\textsubscript{4}PteGlu were dissolved in 1 ml of H\textsubscript{2}O, and the concentration was determined by using the molar extinction coefficient of 5-formyltetrahydrofolate at 287 nm (\(\varepsilon = 31,500\) M\(^{-1}\) cm\(^{-1}\)) (14). A solution of 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu was added to the extract as an ATP carrier before injection into the HPLC.

Synthesis of \(\textsuperscript{18O}\)-labeled 5-Formyltetrahydrofolate—The labeled 5-CHO-H\textsubscript{4}PteGlu was synthesized by first converting the \(\textsuperscript{18O}\)-labeled 5-CHO-H\textsubscript{4}PteGlu to 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu in acid and then hydrolyzing back to 5-CHO-H\textsubscript{4}PteGlu in H\textsubscript{2}\[18O\]. (6S)-5-CHO-H\textsubscript{4}PteGlu (1.9 \(\mu\)mol) was dissolved in 0.4 ml of H\textsubscript{2}\[18O\] in a 1.5-ml Eppendorf tube, and the pH was adjusted to 1 by adding 10 \(\mu\)l of 6 N HCl. The solution was kept at 4 °C, and the conversion to 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu was monitored at 360 nm. After 48 h, 64% of the (6S)-5-CHO-H\textsubscript{4}PteGlu had been converted to 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu. The solution was then made 150 mM in potassium phosphate, pH 6.6, with the addition of solid K\textsubscript{2}HPO\textsubscript{4}. The solution was also made 100 mM in 2-mercaptoethanol. The sealed Eppendorf tube was then heated at 90 °C for 5 h to convert the 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu to (6S)-5-CHO-H\textsubscript{4}PteGlu (17). The yield of (6S)-5-CHO-H\textsubscript{4}PteGlu, calculated from the absorbance at 288 nm, was greater than 95%. This concentration was confirmed by the ability of 5-CHO-H\textsubscript{4}PteGlu to form a quinonoid complex absorbing at 502 nm with serine hydroxymethyltransferase and glycine (17). The large amount of phosphate in the product solution was then removed by chromatography on a 0.5 \(\times\) 20-cm BioGel P2 column equilibrated with 5 mM NH\textsubscript{4}HCO\textsubscript{3}. The fractions with maximum absorbance at 288 nm were pooled and lyophilized to dryness. Based on the amount of 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu formed in acid from the initial (6S)-5-CHO-H\textsubscript{4}PteGlu, the product contained 36% \(\textsuperscript{18O}\)- and 64% \(\textsuperscript{16O}\)-labeled 5-CHO-H\textsubscript{4}PteGlu.

NMR Determination of \(\textsuperscript{18O}\)-Phosphate—A solution of 0.5 ml was placed in a 5 \(\times\) 180-mm NMR tube and contained the following reagents: 50 mM K MES, pH 6.0, 5 mM MgATP, 2.0 \(\mu\)M (6S)-5-CHO-H\textsubscript{4}PteGlu, and 90% D\textsubscript{2}O as an internal frequency lock. A \(\Delta^1\)P spectrum was obtained as background before the addition of MS at 202.36 MHz on a Varian Gemini-500 spectrometer at 30 °C with 384 transients, a 1.94-s acquisition time, and 30-\(\mu\)s pulse width. To the reaction solution, 2.4 \(\mu\)M of MS was added, and the solution was incubated for 1 h at 30 °C. Then, a second \(\Delta^1\)P spectrum was acquired using the same parameters as described above.

Rapid Reaction Kinetics—The rate of formation of 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu, from either 5-CHO-H\textsubscript{4}PteGlu or 5-CHO-H\textsubscript{4}PteGlu in the presence of excess MS was determined by measuring the increase in A\textsubscript{287} in a stopped-flow spectrophotometer from Kinetic Instruments Inc. A solution of 11.6 \(\mu\)M MS in 20 mM K MES, pH 6.0, was flowed against 24 \(\mu\)M (6R,6S)-5-CHO-H\textsubscript{4}PteGlu or 11 \(\mu\)M (6S)-5-CHO-H\textsubscript{4}PteGlu in the same K MES buffer. The biphase time course reaction profile was fitted by the Marquardt-Levenberg double exponential model, which gave the first-order rate constants and amplitudes of each phase. The experiments were performed at 21 °C.

**RESULTS**

Substrate Activity of 5-CHO-Tetrahydrohomofolate—5-CHO-tetrahydrohomofolate differs from 5-CHO-H\textsubscript{4}PteGlu by one additional methylene group between the pteridine and aminobenzoyl rings. To form the product 5,11-CH\textsubscript{1}H\textsubscript{4}PteGlu, the enzyme was saturated with 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu rather than the 5-membered ring of 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu. However, 5,11-CH\textsubscript{1}H\textsubscript{4}PteGlu has been previously synthesized and shown to be an inhibitor of the enzyme 5,10-methylenetetrahydrofolate cyclohydrolase, which converts 5,10-CH\textsubscript{1}H\textsubscript{4}PteGlu to 5-CHO-H\textsubscript{4}PteGlu (18). We determined that 5,11-CH\textsubscript{1}H\textsubscript{4}PteGlu could be formed by MS catalysis from 5-CHO-H\textsubscript{4}PteGlu by observing spectral changes at 316 nm, which characterizes the presence of 5,11-CH\textsubscript{1}H\textsubscript{4}PteGlu (14). Less than 10% of 5 \(\mu\)M 5-CHO-tetrahydrofolate was converted to 5,11-CH\textsubscript{1}H\textsubscript{4}PteGlu after incubation with 0.6 \(\mu\)M of MS in 20 mM K MES buffer at 30 °C for 1 h. Because of the low turnover rate, it was not possible to determine an accurate K\textsubscript{m} value for this compound. However, the rate did not change with increasing substrate concentration, suggesting that the K\textsubscript{m} value was much less than 5 \(\mu\)M. Assuming the enzyme was saturated with 5-CHO-tetrahydrofolate in these assays, an apparent K\textsubscript{m} value of 0.005 s\(^{-1}\) was determined from the initial velocity, which is 0.1% of the K\textsubscript{m} value of 5-CHO-H\textsubscript{4}PteGlu.
Evidence for Irreversibility of Reaction I—Reversibility of the MS-catalyzed reaction had previously been examined by observing the formation of 5-CHO-H₄PteGlu from 5,10-CH₄-H₄PteGlu. No 5-CHO-H₄PteGlu could be detected, suggesting that the reaction was not reversible (6). However, when 5-CHO-tetrahydrohomofolate was used, a linear incorporation of [³H]ADP into ATP was observed with a k_{ox} of 66 s⁻¹ (Fig. 1). About 12.5% ADP was converted into ATP in 120 min in the presence of 5-CHO-tetrahydrohomofolate compared to 1% for (6R,6S)-5-CHO-H₄PteGlu.

The exchange experiments were also performed with increasing concentrations of MgADP at saturating levels of 5-CHO-H₄PteGlu. The first 30 min of radiolabel incorporation into ATP were measured. The results show that the conversion of ADP to ATP depends on the initial concentration of ADP (Fig. 2). A double reciprocal plot of initial rate of [³H]ATP formation versus the concentration of ADP is linear, giving an apparent K_m of 1.4 mM for ADP and a K_m for the formation of ATP of 70 s⁻¹. A product inhibition constant of 0.3 mM for ADP has been previously determined (7).

[³P]Phosphate NMR Studies—The enzymatic mechanism of coupling ATP to the conversion of substrates to products can often be determined by following the path of oxygen atoms present in the substrate (20, 21, 27). To follow these previously used methods, 5-CH[¹⁸O]-H₄PteGlu was synthesized as described under “Experimental Procedures.” Using [¹⁸O]ATP, Reaction I was performed in an NMR tube. The product phosphate was then analyzed by [³P]NMR spectroscopy. The presence of one [¹⁸O]phosphate results in a 0.02 ppm up-field shift in the [³P]signal. (20). Fig. 3 shows that both [¹⁸O]phosphate (34%) and singly labeled [¹⁸O]phosphate (66%) were formed. The substrate was known to contain about 36% 5-CH[¹⁵O]-H₄PteGlu and 64% 5-CH[¹⁸O]-H₄PteGlu. These results suggest that the oxygen atom on the formyl group of 5-CHO-H₄PteGlu was transferred quantitatively to phosphate.

Rotamer Specificity—In solution, 5-CHO-H₄PteGlu exists as two slowly interconverting rotamers arising from partial hindered rotation of the formyl-N₅ formamide bond. These two rotamers are present at a ratio of 2.35:1 at 25 °C (22, 23). The structures of both rotamers have previously been determined by 13C and 1H NMR spectroscopy. The more abundant rotamer has the formyl carbonyl oriented toward H₆ and the formyl proton oriented in the same plane as the keto group of C₄ of the pteridine ring.

If only one rotamer of 5-CHO-H₄PteGlu serves as a substrate for MS, the presence of excess enzyme should rapidly convert this rotamer to 5,10-CH⁻⁻H₄PteGlu with conversion of the second rotamer being controlled by the much slower nonenzymatic interconversion of the two rotamers. Using stopped-flow spectrophotometry, the rate of product formation can be fol-
of both 5-CHO-H4PteGlu and 5-CHO-H4PteGlu5. This is the
substrate used by MAS, which uses the more abundant rotamer
determined by NMR spectroscopy (23). These results suggest
is also very close to the equilibrium ratio (2.35:1 at 25 °C)
determined previously on our instrument (25). The ratio of the
experimental error, the same rate of rotamer interconversion
based on the initial concentration of 5-CHO-H4PteGlu5. After subtracting
the residual phosphate peak from panel A, the newly formed peaks
in panel B represent 34% [O16]phosphate and 66% singly labeled
[O18]phosphate.

Varying with the use of excess enzyme. Fig. 4 shows the rate of
5,10-CH0-H4PteGlu5 formation at 360 nm when 11.6 μM MS
was flowed against 11.7 μM 5-CHO-H4PteGlu5 and 1.9 mM
MgATP at 21 °C. The data were fitted to a double exponential
equation as represented by the solid line. The slower rate was 0.03 s
and was the same when either 5-CHO-H4PteGlu or
5-CHO-H4PteGlu5 was used as substrate. This rate is, within
experimental error, the same rate of rotamer interconversion
previously determined on our instrument (25). The ratio of the
amplitudes for the rapid and slow phases was 2.5. This result is
also very close to the equilibrium ratio (2.35:1 at 25 °C)
determined by NMR spectroscopy (23). These results suggest
that MS uses as the substrate only the more abundant rotamer
of both 5-CHO-H4PteGlu and 5-CHO-H4PteGlu5. This is the
same rotamer bound by dihydrololate reductase (24) and serine
hydroxymethyltransferase (25).

**DISCUSSION**

The metabolite 5,10-CH0-H4PteGlu5, can be hydroydized to
both 10-CHO-H4PteGlu and 5-CHO-H4PteGlu5, at neutral pH.
Its K_{eq} for formation of 10-CHO-H4PteGlu5 can be readily
determined experimentally, but the K_{eq} for formation of
5-CHO-H4PteGlu5 can only be determined under acidic conditions
and is less accurate. Using the K_{eq} experimentally determined
by Kay et al. (2) for Reaction II, we can calculate that the
equilibrium ratio of product to reactant at pH 7.0 is 1:6.5 × 10^{-3}.

5-CHO-H4PteGlu5 + H2O → 5,10-CH0-H4PteGlu5

**REACTION II**

Coupling ATP hydrolysis to Reaction II results in Reaction I
with a predicted K_{eq} of 66 M. A similar calculation, based on
the tetrahydroquinoline analog of 5-CHO-H4PteGlu5, indicates
that the K_{eq} for Reaction I would approach 500 M (2, 9).
Our failure to find any incorporation of labeled ADP into ATP
puts an upper limit on the rate of the reverse of Reaction I of
0.08 min^{-1}. This is nearly 4000-fold slower than the enzyme-
catalyzed rate of the forward reaction. This suggests that at pH
7.0 the equilibrium of Reaction II does not favor 5-CHO-H4PteGlu5,
to the extent predicted by the studies of Kay et al.,
since the predicted K_{eq} of 66 M for Reaction I would show some
isotope exchange of ADP into ATP.

The studies of non-equilibrium isotope exchange of ADP and
ATP with both 5-CHO-H4PteGlu and 5-CHO-tetrahydrofolate
suggest that although the overall reaction is irreversible,
there is a reversible step involved on the pathway to the
formation of 5,10-CH0-H4PteGlu5. Scheme I proposes a mecha-
nism to explain the observed results. The major rotamer of the
enolate form of 5-CHO-H4PteGlu5 (structure I) makes a nucleo-
philic attack on the γ-phosphoryl group of ATP to form a
phosphorylated 5-CHO-H4PteGlu5 (structure II, Scheme I).
At this intermediate, enzyme-bound ADP can freely equilibrate
with solvent ADP. Reversal of this reaction (II to I) would
account for the incorporation of [3H]ADP into ATP. Fig. I.
Intermediate II would be formed with both 5-CHO-H4PteGlu
and 5-CHO-tetrahydrofolate. The studies with varied amounts of ADP
show saturation kinetics (Fig. 2). The linear
double reciprocal plot suggests a Michaelis-Menten mechanism
in which the K_{m} for ADP is 1.4 mM and the rate from ADP to
ATP is about 70 s^{-1}. This rate is essentially the same rate
determined from the study of ADP and ATP isotope exchange
in the presence of 5-CHO-tetrahydrofolate (66 s^{-1}). The
rate of conversion of intermediate II to I is 10 times the k_{cat}
of 5 s^{-1} determined previously for Reaction I (6).

Intermediate II can proceed further in the reaction by attack
of N10 of 5-CHO-H4PteGlu5 to form a putative tetrahedral inter-
mediate (structure III, Scheme I). This would collapse to
eliminate phosphate and form the product 5,10-CH0-H4PteGlu5
(structure IV). It would be this last step that may be essentially
irreversible since phosphate would be a poor nucleophile in the
back reaction. The value of k_{cat} probably is determined by
either the conversion of intermediate II to III or the elimination
of phosphate to form product. The observation that k_{cat}
for 5-CHO-tetrahydrofolate is 1000-fold less than the K_{eq},
for 5-CHO-H4PteGlu5 suggests that it is the conversion of interme-
diate II to intermediate III. The N11 of the homofolate analog
would probably be out of optimum position to make the nucleo-
philic attack on the phosphorylated intermediate II.

The NMR study provides additional evidence for the formation
of the phosphorylated intermediate II. The mechanism in
Scheme I predicts that the formyl oxygen of 5-CHO-H4PteGlu
would be quantitatively transferred to phosphate. Starting the
reaction with 5-[18O]CHO-H4PteGlu5, NMR analysis shows that

\[^2\text{K_{eq}}\text{is the value of the equilibrium constant with the H}_{2}\text{O}^{-}\text{concentration adjusted to pH 7.0.}\]
the oxygen is transferred to phosphate. An alternative mechanism, which would be consistent with the NMR data, is that ATP forms a phosphorylated enzyme intermediate and that 5-CHO-H₄PteGlu is phosphorylated by the phosphoenzyme. However, this mechanism suggests that the enzyme would catalyze the equilibration of [³H]ADP and ATP in the absence of the 5-CHO-H₄PteGlu. We found no evidence for this substrate-independent ADP-ATP exchange, which argues against a phosphorylated enzyme being formed.

Another folate-dependent enzyme that utilizes ATP is N⁵⁰-formyltetrahydrofolate synthetase, which catalyzes the synthesis of 10-formyltetrahydrofolate from formate and tetrahydrofolate. ATP hydrolysis to ADP and phosphate is coupled to this reaction to shift the equilibrium to favor 10-CHO-H₄PteGlu. In eukaryotic cells, this activity is part of a trifunctional enzyme, while in bacteria it is monofunctional (26). Mejillano et al. (26) demonstrated that both the prokaryotic and eukaryotic enzymes form a formyl phosphate as an intermediate. However, ADP remains bound to the enzyme so the formate-dependent ADP-ATP exchange is very slow. Schrimsher et al. (27) have shown that aminimidazole ribonucleotide synthetase catalyzes the ATP-dependent formation of the imidazole ring in purine biosynthesis. Starting with [¹⁵O]formylglycinamidine ribonucleotide, these authors showed the existence of a phosphorylated intermediate of the substrate. However, ADP-ATP exchange was only 1/20th the rate of the reaction, again probably the result of a slow release of ADP from the enzyme.

Addendum—During the review of this manuscript, a communication by Kounga et al. (28) was published, which presented similar ³²P NMR data as shown in Fig. 3.

REFERENCES
1. Peter, J. M., and Greenberg, D. M. (1958) J. Am. Chem. Soc. 80, 2719–2722
2. Kay, L. D., Osborn, M. J., Hateri, Y., and Huennekens, F. M. (1960) J. Biol. Chem. 235, 195–201
3. Greenberg, D. M., Wynston, L. K., and Nagabushanam, A. (1965) Biochemistry 4, 1872–1878
4. Grimshaw, C. E., Henderson, G. B., Soppe, G. G., Hansen, G., Mathur, E. J., and Huennekens, F. M. (1964) J. Biol. Chem. 239, 2728–2733
5. Bertrand, R., Mackenzie, R. E., and Jolivet, J. (1971) Biochim. Biophys. Acta 2728–2733
6. Hopkins, S., and Schirch, V. (1984) J. Biol. Chem. 259, 5618–5622
7. Maras, B., Stover, P., Valiante, S., Barra, D., and Schirch, V. (1994) J. Biol. Chem. 269, 18429–18433
8. Stover, P., and Schirch, V. (1993) Trends Biochem. Sci. 18, 102–106
9. Benkovic, S. J., Bullard, W. P., and Bendovic, P. A. (1972) J. Am. Chem. Soc. 94, 7542–7549
10. Kruschwitz, H. L., McDonald, D., Cassins, E. A., and Schirch, V. (1994) J. Biol. Chem. 269, 28757–28763
11. Machover, D., Schwarzenberg, L., Goldschmidt, B., Hayat, M., Dorval, T., Misset, J. L., Jasmin, C., Maral, R., and Mathe, G. (1982) Cancer Treat. Rep. 66, 1803–1907
12. Schretzer, B. I., Dicker, A. P., and Bertino, J. R. (1990) FASEB J. 4, 2441–2452
13. Bertrand, R., and Jolivet, J. (1989) J. Biol. Chem. 264, 8843–8846
14. Temple, C., and Montgomery, J. A. (1984) in Chemistry and Biochemistry of Folate, (Blakely, R. L., and Benkovic, S. J., eds) Vol. I, p. 80, John Wiley & Sons, Inc., New York
15. Stover, P., Huang, T., Schirch, V., Maras, B., Valiante, S., and Barra, D. (1993) in Chemistry and Biology of Pteridines and Folate (Ayling, J. E., Nair, M. G., and Baugh, C. M., eds) pp. 723–726, Plenum Press, NY
16. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–663
17. Stover, P., and Schirch, V. (1992) Anal Biochem. 202, 82–88
18. Donanico, P., and Benkovic, S. J. (1981) J. Med. Chem. 24, 1086–1088
19. May, M., Bardase, T. J., Barger, F. L., Lanford, M., Ravel, J. M., Sutherland, G. L., and Shive, W. (1951) J. Am. Chem. Soc. 73, 3067–3069
20. Cohen, M., and Hu, A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 200–203
21. Seddon, A. P., and Mester, A. (1986) J. Biol. Chem. 261, 11538–11543
22. Feeney, J., Albrand, J. P., Boicelli, C. A., Charlton, P. A., and Yourg, D. W. (1980) J. Chem. Soc. Perkin Trans. 1, 166–170
23. Poe, M., and Benkovic, S. J. (1989) Biochemistry 28, 4576–4583
24. Feeney, J., Birdsall, B., Albrand, J. P., Roberts, G. C., Burgen, A. S. V., Charlton, P. A., and Yourg, D. W. (1980) J. Biol. Chem. 259, 11538–11543
25. Mejillano, M. R., Jahansouz, H., Matsunaga, T. O., Kenyon, G. L., and Himes, R. H. (1989) Biochemistry 28, 5136–5145
26. Schrimsher, J. L., Schentiel, F. J., and Stubble, J. (1986) Biochemistry 25, 4356–4365
27. Kounga, K., Vander Velde, D. G., and Himes, R. H. (1995) FEBS Lett. 364, 215–217