^{18}Oxygen incorporation into inorganic phosphate in the reaction catalyzed by $N^{5,10}$-methenyltetrahydrofolate synthetase

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Abstract **The mechanism of the $N^{5,10}$-methenyltetrahydrofolate synthetase reaction was probed by determining the source of the oxygen atom introduced between the β- and γ-phosphates as ATP is converted to ADP and P_i. The reaction was performed using a mixture of [^{18}O]- and [^{16}O]N^5-formyltetrahydrofolate in the presence of [^{16}O]H_2O and using [^{16}O]N^5-formyltetrahydrofolate in the presence of a 1:1 mixture of [^{18}O]H_2O and [^{16}O]H_2O. ^{31}P NMR spectroscopy was used to examine the products. It was found that ^{18}O from the formyl group was incorporated into P_i, and that ^{18}O was not incorporated from the solvent. The results are consistent with a mechanism involving phosphorylation of the formyl group at the N^5-position, followed by displacement of the phosphate by the 10-nitrogen.**

Key words: $N^{5,10}$-Methenyltetrahydrofolate synthetase; ^{18}O incorporation; Enzyme mechanism

1. Introduction

N^5-Formyltetrahydrofolate has been used for a number of years to rescue cancer patients from a tetrahydrofolate deficiency after treatment with methotrexate. In addition, this folate derivative is known to potentiate the anti-tumor effects of 5-fluorouracil [1]. The enzyme $N^{5,10}$-methenyltetrahydrofolate synthetase is responsible for both of these effects by bringing the N^5-formyl derivative into the metabolic pool (Eqn. 1) [2].

$$N^5\text{-CHO-H}_4\text{folate} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} N^{5,10}\text{-methenyl-H}_4\text{folate} + \text{ADP} + P_i \quad (1)$$

N^5-Formyltetrahydrofolate also occurs naturally as one member of the pool of folate one-carbon units, probably arising from the hydrolysis of $N^{5,10}$-methenyltetrahydrofolate by serine hydroxymethyltransferase, a second catalytic activity of the latter enzyme [3]. Although in the past it was thought that N^5-formyltetrahydrofolate was inert as a normal metabolic intermediate, it is now thought to play a metabolic role [4].

The mechanism of the synthetase has not been thoroughly investigated. A plausible mechanism involves phosphorylation of the N^5-formyl group by ATP followed by displacement of the phosphate by the 10-nitrogen (Fig. 1) [2]. This mechanism predicts transfer of the formyl oxygen to the leaving γ-phosphate of ATP. We investigated this possibility using ^{18}O-labeled substrate and ^{31}P NMR spectroscopy.

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2. Materials and methods

$N^{5,10}$-Methenyltetrahydrofolate synthetase has been purified from bacterial [5] and animal [6,7] sources. We used cow liver as a source and followed a modification of the procedure of Bertrand et al. [7] to purify the enzyme. The steps included ammonium sulfate precipitation and successive chromatographic steps using hydroxylapatite, matrex-red A, and folinate-Sepharose. The enzyme preparation contained no ATPase activity measured over a 20 h period. The resulting protein showed a single band on SDS-PAGE with a molecular mass of 25 kDa and had a k_{cat} value of 3.5 min^{-1}. N^{10}-Formyl-tetrahydrofolate synthetase was purified from *E. coli* strain JM109 transformed with a Bluescript vector carrying the *Clostridium cylindrosporum* gene for the enzyme [8].

N^5-Formyltetrahydrofolate containing ^{18}O in the formyl group was synthesized by hydrolyzing $N^{5,10}$-methenyltetrahydrofolate in 50% [^{18}O]H_2O. A solution of 220 mM N^5-formyltetrahydrofolate in 50 mM 2-mercaptoethanol was converted to the methenyl derivative by adjusting the pH to 2 with 6 NHCl and incubating for 1 h at room temperature [9]. The solution was taken to dryness. To prepare ^{18}O-containing N^5-formyltetrahydrofolate, 70 mg of the methenyl compound was added to 2 ml of 50% [^{18}O]H_2O containing 50 mM 2-mercaptoethanol and 0.1 M Na_2CO_3 adjusted to pH 12 with NaOH and preheated at 100°C (10). The solution was heated for an additional 1 h at 100°C. After adjusting the pH to 8, the product was purified on DEAE-cellulose [11], lyophilized and chromatographed on Biogel-P2 to remove salts. The ^{13}C NMR signal of the N^5-formyl group shown in Fig. 2 contains a doublet consistent with an ^{18}O content of about 43%. The two signals are separated by 0.026 ppm, the expected difference [12]. [^{16}O,^{18}O]Formic acid was prepared by incubating formic acid 98% [^{18}O]H_2O (Cambridge Isotope Laboratories) in 0.3 M HCl in a sealed tube at 140°C for 7 days. The ^{13}C NMR spectrum showed three signals for [$^{16}O_2$]-, [^{16}O,^{18}O]-, and [$^{18}O_2$]formic acid separated by about 0.025 ppm.

The N^{10}-formyltetrahydrofolate synthetase reaction was carried out in a 1 ml volume containing 0.1 M triethanolamine, pH 8.0, 0.1 M 2-mercaptoethanol, 5 mM ATP, 10 mM $MgCl_2$, 2 mM (*R,S*)-tetrahydrofolate, 50 mM KCl, 40 mM sodium formate, and 30 μg enzyme. The sodium formate was a mixture of the [^{16}O,^{18}O]formate synthesized above and [$^{16}O_2$]formate such that the final ^{18}O content of the total formate was about 50%. After incubation for 40 min at 37°C the solution was placed on ice and added to a small column containing 200 mg of activated charcoal sandwiched between 1 ml volumes of Chelex 100 (Bio-Rad). The column was centrifuged at 1500 rpm for 5 min and ashed with another 2 ml of H_2O. The combined eluants were lyophilized and the dried material suspended in 0.5 ml H_2O containing 10% D_2O and 2 mM EDTA. The pH was adjusted to 8 with KOH. The final solution contained 0.8 mM P_i.

The $N^{5,10}$-methenyltetrahydrofolate synthetase reaction was carried out in a 1 ml volume containing 50 mM MES, pH 6, 5 mM ATP, 12 mM $Mg(OAc)_2$, 5 mM [^{16}O,^{18}O]N^5-formyltetrahydro folate containing 43% ^{18}O, 14 mM 2-mercaptoethanol, and 30 μg enzyme. Incubation was for 90 min at 37°C. The reaction was cooled on ice and the P_i recovered from a charcoal-Chelex-100 column as described above. The reaction was also done in 50% [^{18}O]H_2O using [^{16}O]N^5-formyltetrahydrofolate.

P_i was determined by a modification of the Fiske-Subbarow method [13] in a total volume of 1 ml. ^{13}C and ^{31}P NMR spectra were recorded at 125.7 MHz and 202.4 MHz, respectively, on a Brucker AM-500 spectrometer. Chemical shifts are reported relative to sodium 3-(trimethylsilyl)propionate d_4 for ^{13}C, and external 85% H_3PO_4 for ^{31}P.

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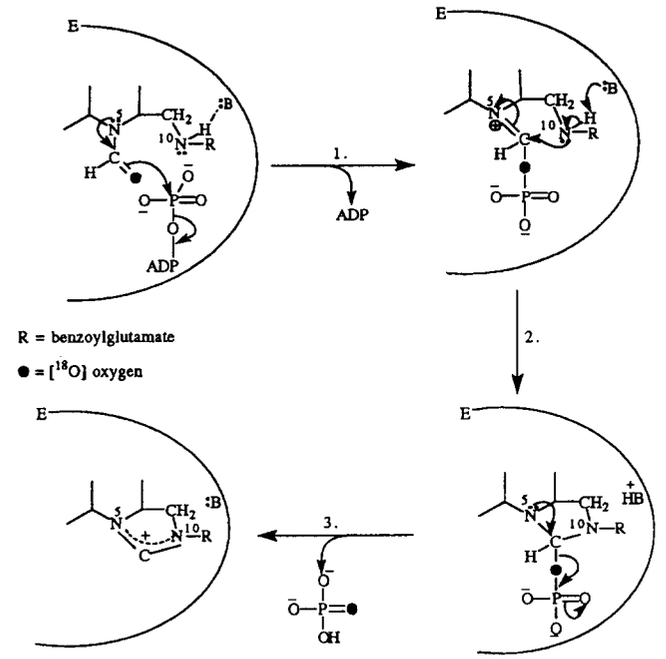

Fig. 1. Proposed catalytic mechanism of $N^{5,10}$-methenyltetrahydrofolate synthetase. The mechanism proposes the formation of an iminium phosphate (reaction 1) which converts to a phosphoimidazolidine (reaction 2) and, after elimination of phosphate, to the methenyl derivative (reaction 3).

3. Results and discussion

As a positive control to ensure that we could detect the formation of a mixture of $[^{16}O_4]P_i$ and $[^{18}O,^{16}O_3]$ P_i, we used the enzyme N^{10}-formyltetrahydrofolate synthetase. The reaction catalyzed by this enzyme involves the phosphorylation of formate by ATP and transfer of the formyl group to nitrogen-10 [14]. In the process one oxygen from formate is incorporated into P_i [15]. The formate used in this experiment contained 50%

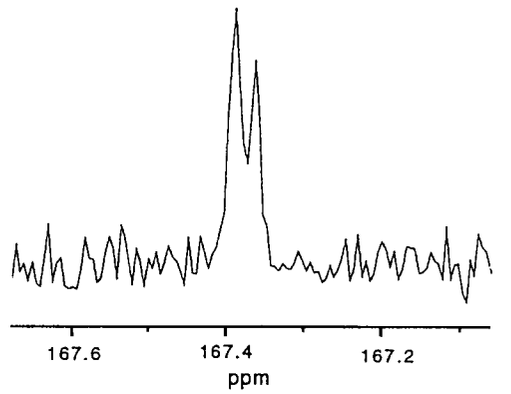

Fig. 2. ^{13}C NMR spectrum of the synthesized $[^{16}O,^{18}O]N^5$-formyltetrahydro folate showing the formyl signal. The solution contained 29 mM of the compound in 10% D_2O at pH 7.

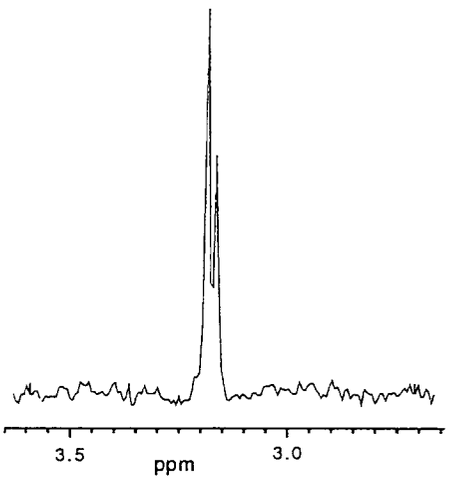

Fig. 3. ^{31}P NMR of the phosphate produced in the N^{10}-formyltetrahydrofolate synthetase reaction performed using $[^{16}O,^{18}O]$formate. The phosphate concentration was 0.8 mM.

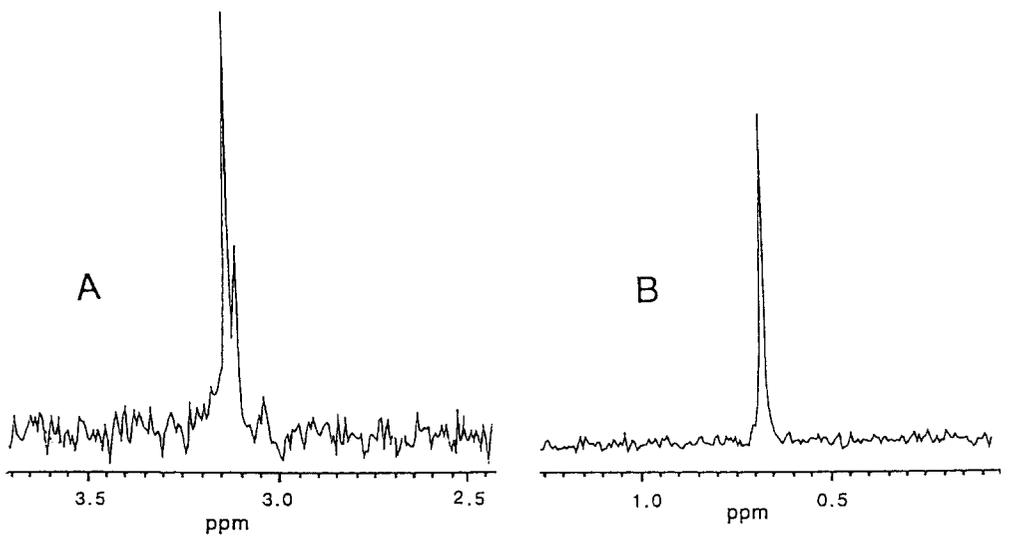

Fig. 4. ^{31}P NMR of phosphate formed in the $N^{5,10}$-methenyltetrahydrofolate synthetase reaction. (A) Signal of the phosphate produced in the reaction performed in $[^{16}O]H_2O$ and using $[^{16}O,^{18}O]N^5$-formyltetrahydro folate. (B) Signal of the phosphate produced in 50% $[^{18}O]H_2O$, 50% $[^{16}O]H_2O$ using $[^{16}O]N^5$-formyltetrahydrofolate. The phosphate concentration was 1.4 mM in A, and 0.9 mM in B. The difference in chemical shift for the P_i signal in A and B is due to a small difference in the pH of the samples. The chemical shift of P_i is sensitive to pH in the pH range of 6–8.

^{18}O. The NMR signal of the P_i formed in the reaction (Fig. 3) appeared as a doublet separated by 0.019 ppm, consistent with the presence of both species of P_i [16]. The content of the [^{18}O,O_3^{16}]isotopomer is 39%. This is lower than the expected value of 50%, and is due to a small contamination of P_i in the ATP.

The N^5-methenyltetrafolate synthetase reaction was performed using a mixture of 43% ^{18}O and 57% [^{16}O]N^5-formyltetrahydrofolate. The P_i formed in this reaction also showed a doublet signal in the NMR spectrum (Fig. 4A). On the other hand, when the reaction was done using [^{16}O]N^5-formyltetrahydrofolate in 50% ^{16}[O]- and 50% ^{18}[O]H_2O, the P_i signal was a singlet (Fig. 4B).

The results of these experiments clearly show that the oxygen atom from the formyl group of N^5-formyltetrahydrofolate is transferred to the leaving γ-phosphate of ATP during the reaction. Solvent oxygen is not incorporated. The data are consistent with a mechanism shown in Fig. 1, in which the formyl group is phosphorylated by ATP to provide a good leaving group for attack by the 10-nitrogen. The mechanism in some respects is similar to that catalyzed by N^{10}-formyltetrahydrofolate synthetase in which ATP is used to phosphorylate formate before attack by the N-10 of tetrahydrofolate.

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References

[1] Keyomarsi, K. and Moran, R.G. (1986) Cancer Res. 46, 5229–5235.
[2] Huennekens, F.M., Henderson, G.B., Vitols, K.S. and Grimshaw, C.E. (1984) Adv. Enzyme Regul. 22, 3–13.
[3] Stover, P. and Schirch, V. (1990) J. Biol. Chem. 265, 14227–14233.
[4] Stover, P. and Schirch, V. (1993) Trends Biochem. Sci. 18, 102–106.
[5] Grimshaw, C.E., Henderson, G.B., Soppe, G.G., Mathur, E.J. and Huennekens, F.M. (1984) J. Biol. Chem. 259, 2728–2733.
[6] Hopkins, S. and Schirch, V. (1984) J. Biol. Chem. 259, 5618–5622.
[7] Bertrand, R., MacKenzie, R.E. and Jolivet, J. (1987) Biochim. Biophys. Acta 911, 154–161.
[8] Rankin, C.A., Haslam, G.C. and Himes, R.H. (1993) Protein Sci. 2, 197–205.
[9] Rowe, P.B. (1980) Methods Enzymol. 66, 733–735.
[10] Zakrzewski, S.F. and Samsone, A.M. (1980) Methods Enzymol. 66, 731–733.
[11] Moran, R.G., Keyomaisi, K. and Colman, P.D. (1986) Methods Enzymol. 122, 309–312.
[12] Riseley, J.M. and Van Etten, R.L. (1980) J. Am. Chem. Soc. 102, 4609–4614.
[13] Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1957) Manometric Techniques, Burgess Press, Minneapolis, p. 272.
[14] Mejillano, M.R., Jahansouz, H., Matsunaga, T.O., Kenyon, G.L. and Himes, R.H. (1989) Biochemistry 28, 5136–5145.
[15] Himes, R.H. and Rabinowitz, J.C. (1962) J. Biol. Chem. 237, 2915–2925.
[16] Cohn, M. and Hu, A. (1978) Proc. Natl. Acad. Sci. USA 75, 200–203.