Volume 332, number 1,2, 150–152 FEBS 13090 October 1993

Solvent oxygen is not incorporated into N^{10}-formyltetrahydrofolate in the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase

Sophie Song, Hossain Jahansouz, Richard H. Himes*

Department of Biochemistry, University of Kansas, Lawrence, KS 66045–2106, USA

Received 23 August 1993

The mechanism of the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase involves the formation of formyl phosphate as an intermediate which then formylates tetrahydrofolate at the N-10 position. Previous studies demonstrated that the non-enzymic formylation of tetrahydrofolate by formyl phosphate occurs exclusively at the more nucleophilic 5-nitrogen in the reduced pyrazine ring. The experiments described in this report were designed to determine whether N^5-formyltetrahydrofolate might be the first product to be formed on the enzyme, followed by formyl transfer to the 10-nitrogen via the cyclic intermediate $N^{5,10}$-methenyltetrahydrofolate. If this were the case, oxygen from solvent H_2O would be incorporated into the formyl group of the N^{10}-derivative. By conducting the reaction in a 1:1 mixture of [^{16}O]H_2O and [^{18}O]H_2O and using ^{13}C NMR spectroscopy we show that no ^{18}O is incorporated into the product and conclude that the reaction proceeds via a direct formylation of the N-10 position by formyl phosphate.

Formyltetrahydrofolate synthetase; Formyltetrahydrofolate; ^{18}O incorporation

1. INTRODUCTION

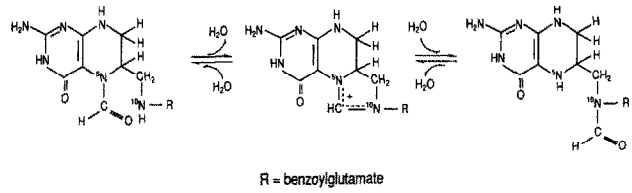
The mechanism of the formylation of tetrahydrofolate catalyzed by N^{10}-formyl-tetrahydrofolate synthetase, EC 6.3.4.3, involves the formation of enzyme-bound formyl phosphate from ATP and formate, followed by transfer of the formyl group to tetrahydrofolate [1,2].

$$HCOO + ATP \rightleftharpoons HC(=O)OPO_3^{=} + ADP \quad (1)$$

$$HC(=O)OPO_3^{=} + H_4folate \rightleftharpoons N^{10}\text{-CHO-}H_4folate + P_i \quad (2)$$

Based on relative nucleophilicities alone, the preferred formylation site should be the 5-nitrogen in the tetrahydropyrazine ring rather than the 10-nitrogen which is part of the *p*-aminobenzoic acid moiety. Non-enzymic formylation of tetrahydrofolate by methyl formate [3], formic acid [4] and formyl phosphate [5] occurs exclusively at the 5 position.

One possible mechanism of the formyl transfer reaction which has not been considered previously, involves the formation of N^5-formyltetrahydrofolate on the enzyme, followed by a transfer to the N-10 position. The interconversion of N^5- and N^{10}-formyltetrahydrofolate through $N^{5,10}$-methenyltetrahydrofolate is a well studied pH-dependent reaction sequence [6].



(3)

If the steps shown in Eqn. 3 do participate in the reaction, incorporation of oxygen from solvent H_2O should occur in the N^{10}-formyl group. By conducting the reaction in 50% [^{18}O]H_2O, equal amounts of [^{16}O] and [^{18}O]N^{10}-formyltetrahydrofolate should be formed. Bonding of an ^{18}O-oxygen to a carbon causes an upfield shift of 0.02 ppm in the ^{13}C NMR spectrum [7]. Thus, if solvent H_2O were incorporated into the product, two signals should be evident in the ^{13}C NMR spectrum of the formyl carbon in the reaction product.

2. MATERIALS AND METHODS

N^{10}-Formyltetrahydrofolate synthetase was purified from *Clostridium cylindrosporum* [2,8] and tetrahydrofolate was prepared by catalytic reduction of folic acid [9]. [^{13}C]Formic acid (99%), [^{18}O]H_2O (98%) and [^{2}H]H_2O (99%) were purchased from Cambridge Isotope Laboratories.

[^{13}C]N^5-Formyltetrahydrofolate was synthesized by incubating 10 mM (6*R*,*S*)-tetrahydrofolate with 100 mM [^{13}C]formic acid at pH 3.8 and room temperature in a 0.5 ml solution which also contained 20% D_2O, 150 mM 2-mercaptoethanol, 20 mM sodium phosphate and

*Corresponding author. Fax: (1) (913) 864 5321.

 Published by Elsevier Science Publishers B.V.

33 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide [10]. After 10 to 15 min, the pH was brought to 8.5 with 10 M KOH. To prepare [^{13}C] N^{10}-formyltetrahydrofolate 12 N HCl instead of 10 M KOH was added to adjust the pH to 1 after the reaction. This step converts the N^5-derivative to $N^{5,10}$-methenyltetrahydrofolate. After 1 h the pH was adjusted to 7.8 with 10 M KOH, converting the cyclic derivative to N^{10}-formyltetrahydrofolate. The progress of these interconversions was followed by monitoring the UV spectra.

The enzyme-catalyzed formation of N^{10}-formyltetrahydrofolate was conducted at 37°C in a 0.5 ml volume containing 25 mM ATP, 27 mM 6(*R,S*)-tetrahydrofolate, 100 mM [^{13}C]formic acid, 10 mM $MgCl_2$, 50 mM KCl, 100 mM triethanolamine · HCl, 56 mM Tris-HCl, 280 mM 2-mercaptoethanol, 20% D_2O, and 450 μg of enzyme. The pH was adjusted to 7.8–8.0 with 10 M KOH. Reaction solutions contained either 100% [^{16}O]H_2O or equal proportions of [^{16}O]- and [^{18}O]H_2O. NMR spectra were taken on a Varian XL-300 spectrometer at 75 MHz. Spectrometer conditions included the following: sweep width, 2,400 Hz; pulse width, 3.5 μs; tip angle, 18.5°; number of transients, 2,000; acquisition time, 2 s. Chemical shifts are reported relative to sodium 3-(trimethylsilyl) propionate d_4 at 0.00 ppm.

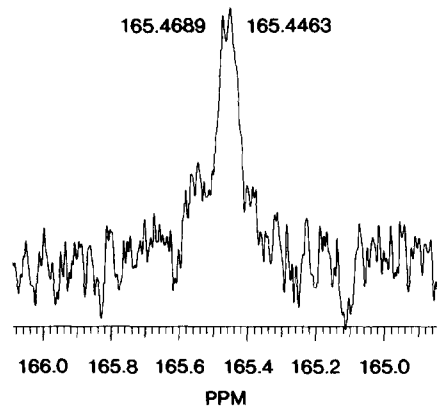


Fig. 1. ^{13}C NMR spectrum of N^{10}-formyltetrahydrofolate formed from $N^{5,10}$-methenyltetrahydrofolate in 50% [^{18}O]H_2O. N^5-Formyltetrahydrofolate was formed from [^{13}C]formic acid and tetrahydrofolate, converted to $N^{5,10}$-methenyltetrahydrofolate with HCl, which upon neutralization, was converted to the N^{10}-formyl derivative. Details are given in section 2.

3. RESULTS AND DISCUSSION

The N^5- and N^{10}-formyl derivatives of tetrahydrofolate, in which the formyl carbon was enriched with ^{13}C, showed ^{13}C signals at 164.562 and 165.461 ppm, respectively, at pH 7.8. To demonstrate that we could detect the presence of N^{10}-formyltetrahydrofolate containing either ^{16}O or ^{18}O in the formyl oxygen we converted $N^{5,10}$-methenyltetrahydrofolate to the N^{10}-formyl derivative in a 1:1 mixture of [^{16}O]- and [^{18}O]H_2O. The ^{13}C NMR spectrum showed that the formyl carbon signal was split into two, at 165.469 and 165.446 ppm (Fig. 1). The 0.02 ppm difference is what would be expected for a ^{13}C-carbon bonded to either ^{16}O or ^{18}O [7].

Because of the interconversions which exist between N^5-formyl-, N^{10}-formyl- and $N^{5,10}$-methenyltetrahydrofolate we were concerned that ^{18}O from solvent could exchange with the formyl oxygen in N^{10}-formyltetrahydrofolate in a non-enzymic process. To test this possibility [^{13}C]N^{10}-formyltetrahydrofolate was incubated in 50% [^{18}O]H_2O. Such an exchange did occur but not within the time periods that were used in the enzyme reaction. After 1.5 h only one signal was observed at 165.465 ppm, but after 30 h two signals were evident, 165.464 and 165.443 (Fig. 2). Because the time necessary for complete formation of product in the enzyme-catalyzed reaction was about 1 min, the slow non-enzymic exchange did not present a problem.

N^{10}-Formyltetrahydrofolate was formed in the enzyme-catalyzed reaction in [^{16}O]H_2O and in a 1:1 mix-

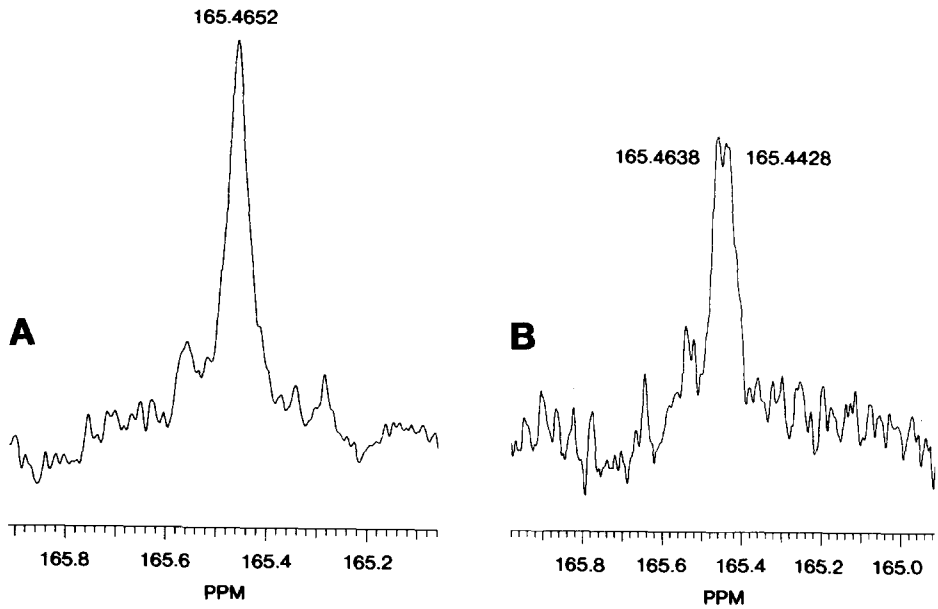


Fig. 2. Non-enzymic exchange of [^{18}O]H_2O with N^{10}-formyltetrahydrofolate. [^{13}C]N^{10}-Formyltetrahydrofolate was synthesized in [^{16}O]H_2O as described in section 2. An equal volume of [^{18}O]H_2O was added and incubation was continued at room temperature for 1.5 h (A) and 30 h (B).

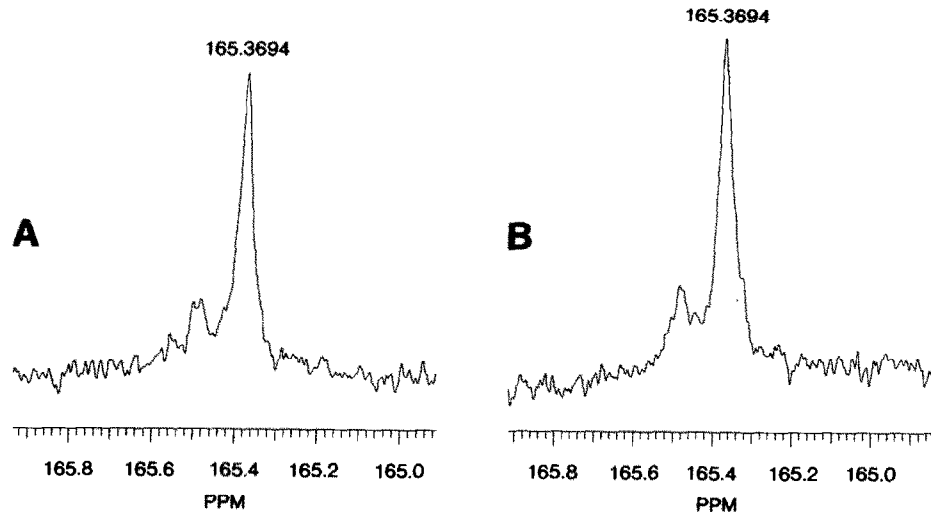


Fig. 3. ^{13}C NMR spectra of N^{10}-formyltetrahydrofolate formed in the enzyme-catalyzed reaction. (A) Spectrum taken immediately after completion of the reaction (1 min). (B) Spectrum taken after a further 45 min incubation at room temperature. Details are given in section 2.

ture of [^{16}O] and [^{18}O]H_2O. The ^{13}C spectrum was examined immediately after the reaction was completed (1 min) and after 45 min. There was no difference in these two spectra. Fig. 3 presents the results of this experiment. Only one signal was observed for the formyl carbon and no difference could be detected in the two spectra, both singlets were at 165.369.

The results of these experiments demonstrate that solvent oxygen is not incorporated into the formyl group of the product, indicating that a direct formylation at the N-10 position occurs. As pointed out previously [5], this suggests that the enzyme may enhance the reactivity of the 10-nitrogen as well as hinder the accessibility of the 5-nitrogen to formylphosphate.

Acknowledgements: This work was supported by NIH Grant DK 07140 and by a grant from the University of Kansas General Research Fund.

REFERENCES

[1] Smithers, G.W., Jahansouz, H., Kofron, J.L., Himes, R.H. and Reed, G.H. (1987) Biochemistry 26, 3943–3948.
[2] Mejillano, M.R., Jahansouz, H., Matsunaga, T.O., Kenyon, G.L. and Himes, R.H. (1989) Biochemistry 28, 5136–5145.
[3] Khalifa, E., Ganguly, A.N., Bieri, J.H. and Viscontini, M. (1980) Helv. Chim. Acta 63, 2554–2558.
[4] Moran, R.G. and Colman, P.D. (1982) Anal. Biochem. 122, 70–78.
[5] Jahansouz, H., Scherübel, D.M. and Himes, R.H. (1990) FEBS Lett. 262, 366–368.
[6] Robinson, D.R. (1971) Methods Enzymol. 18, 716–725.
[7] Risley, J.M. and Van Etten, R.L. (1980) J. Am. Chem. Soc. 102, 4609–4614.
[8] Staben, C., Whitehead, T.R. and Rabinowitz, J.C. (1987) Anal. Biochem. 162, 257–264.
[9] Samuel, C.E., D'Ari, L. and Rabinowitz, J.C. (1970) J. Biol. Chem. 245, 5115–5121.
[10] Moran, R.G., Keyomarsi, K. and Colman, P.D. (1986) Methods Enzymol. 122, 309–312.