Volume 262, number 2, 366–368 FEBS 08276 March 1990

Formylation of tetrahydrofolate by formyl phosphate

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Received 5 February 1990

Formyl phosphate is the putative intermediate in the formylation of tetrahydrofolate (THF) catalyzed by N^{10}-formylTHF synthetase. In this study the non-enzymic reaction between formyl phosphate and THF was examined at 5°C. ^{1}H-NMR, HPLC and kinetic analysis of the proton-catalyzed conversion of the product to $N^{5,10}$-methenylTHF were used to identify the product. In contrast to the enzyme reaction, which produces N^{10}-formylTHF, N^5-formylTHF was the only formylated THF derivative formed. The reaction was conducted at pH values of 3, 5, and 7, with the highest yield being obtained at pH 5 (64–85%, based on THF). The enzyme, therefore, changes the regioselectivity of this reaction by increasing the reactivity of the 10-nitrogen and either decreasing the reactivity of the 5-nitrogen or limiting its accessibility to formyl phosphate. 2-Mercaptoethanol, present in the reaction mixture to protect THF from O_2, was also formylated by formyl phosphate, at the oxygen position.

Formyl phosphate; Tetrahydrofolate; Formyltetrahydrofolate synthetase, N^{10}-

1. INTRODUCTION

Evidence has accumulated in recent years which indicates that formyl phosphate is an enzyme-bound intermediate in the formylation of tetrahydrofolate (THF) catalyzed by N^{10}-formylTHF synthetase.

$$MgATP + HCOO^- + THF \rightleftharpoons MgADP + N^{10}\text{-formylTHF} + P_i$$

This evidence includes the facts that the enzyme can utilize formyl phosphate to formylate THF and phosphorylate ADP [1], the rate constant for formylation of THF is consistent with formyl phosphate being a kinetically competent intermediate [2], and the enzyme catalyzes a slow synthesis of formyl phosphate from ATP and formate [2]. To learn more about the feasibility of formyl phosphate as an intermediate in the enzyme reaction, we decided to study the non-enzymic reaction between the putative intermediate and THF. As described in this report, we have found that the reaction between THF and formyl phosphate leads to formylation on the 5 nitrogen, in contrast to the enzymic formylation of the 10 nitrogen.

2. MATERIALS AND METHODS

Formyl phosphate was synthesized as described by Smithers et al. [1], except that the temperature used in preparing the intermediate, formyl fluoride, was 90–95°C. THF was prepared from folic acid and purified as described by Samuel et al. [3] except that 0.15 M $NaHCO_3$ was used as the buffer to elute THF from DEAE-cellulose rather than Tris-HCl, which would have interfered in NMR spectra. N^5-FormylTHF was purchased from Sigma Chemical. $N^{5,10}$-MethenylTHF was made by incubating N^5-formylTHF in 0.1 M HCl/0.1 M 2-mercaptoethanol at 50°C for 2 h. N^{10}-FormylTHF was formed by dissolving the methenyl derivative in 0.1 M 2-mercaptoethanol, pH 8, and keeping the solution at room temperature for 1 h.

Reactions were carried out at 5°C using a 5:1 molar ratio of formyl phosphate to THF in the presence of 0.1 M 2-mercaptoethanol and 0.15 M $NaHCO_3$. (The presence of 2-mercaptoethanol as a reductant is necessary because of the oxygen lability of THF.) For examination of the products by ^{1}H-NMR, the reaction was run in 99.8% D_2O and the initial concentration of THF was 20 mM. ^{1}H-NMR spectra were taken during the course of the reaction at 0, 4, 8, 12 and 24 h on a Varian XL-300 spectrophotometer. 3-(Trimethylsilyl)-1-propanesulfonate (DSS), 1 mM, was added as an internal standard. For examination by HPLC, the reactions were run with 5 mM THF and samples were removed at time periods up to 24 h, frozen and stored at −80°C. HPLC was performed using a Beckman Ultrasphere IP column (4.6 mm × 15 cm). The two solutions used were 5 mM tetrabutylammonium phosphate (TBAP) in 5 mM 2-mercaptoethanol (A), and 50% ethanol containing 5 mM TBAP and 5 mM 2-mercaptoethanol (B). The elution gradient was 15% B for 10 min, increasing to 20% B over 25 min, then increasing to 25% B over 15 min [4]. Solution variables included pD (3.0, 5.0, 7.0), organic solvent (50% acetonitrile and 50% dimethylsulfoxide) and the presence of Mg^{2+} (0.01 M and 0.1 M $MgCl_2$).

3. RESULTS AND DISCUSSION

The ^{1}H-NMR spectra of the standard compounds, THF, N^5-formylTHF, and N^{10}-formylTHF, showed the expected resonance signals as reported in the literature [5–7], including both conformational isomers of the N^5-formyl derivative. During the course of the reaction a signal at 8.60 ppm appeared, presumably due to the formyl proton of N^5-formylTHF (table 1). Absent was the 8.55 ppm signal due to the formyl proton of N^{10}-formylTHF. That the 8.60 ppm signal was due to N^5-formylTHF was shown by adding an equal

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Table 1

Comparison of properties of N^5-formylTHF, N^{10}-formylTHF, and the product of the reaction between formyl phosphate and tetrahydrofolate

Compound	^{1}H-NMR C(O)H (ppm)	HPLC elution time (min)	Cyclization reaction (k(obs), min^{-1})
N^{10}-formylTHF	8.55	22	1.24
N^5-formylTHF	8.60	33	0.42
Product	8.60	33	0.40

concentration of N^5-formylTHF or N^{10}-formylTHF to the product. In the former case only one signal was observed at around 8.60 ppm and in the latter case two signals were present. At the end of 24 h the doublet at 8.50 ppm due to formyl phosphate, had decreased by about 95%. ^{31}P-NMR of products formed at pD 5 failed to show the occurrence of a signal for an N-P bond (10 ppm). HPLC analysis of the reaction products also indicated that N^5-formylTHF was the only THF derivative that was formed (table 1).

During the reaction an unexpected ^{1}H-NMR signal was observed at 8.19 ppm. This signal was identified as the formyl proton in 2-mercaptoethylformate, the O-formylated product of 2-mercaptoethanol, by reacting formyl phosphate with 2-mercaptoethanol, ethylene glycol and ethane dithiol. Signals observed were: ethane dithiol, 6.63 ppm; ethylene glycol, 8.19 ppm; and 2-mercaptoethanol, 8.18 ppm (major), 6.90 ppm (minor).

As an additional proof that the formylation of THF by formyl phosphate occurred on the N^5 position, the rate of the proton-catalyzed cyclization of the product to methenylTHF was determined. It is known that N^{10}-formylTHF cyclizes at a significantly faster rate than N^5-formylTHF. The reaction was initiated by adjusting the solution to 0.63 M in HCl by the addition of a small volume of 12 M HCl. The formation of the cyclic derivative was followed by measuring the increase in absorbance at 350 nm (ϵ = 24900 M^{-1} cm^{-1}) [8] and first order rate constants were determined at room temperature. The observed rate constants presented in table 1 provide further evidence that the formylated THF product is the N^5 derivative.

The yields of the products of the reaction at different pH values after 24 h determined by ^{1}H-NMR and HPLC are presented in table 2. The reaction system is complex with 3 reactions taking place: hydrolysis of formyl phosphate, formylation of THF and formylation of 2-mercaptoethanol. In a separate experiment the yield of N^5-formylTHF was determined after conversion to the $N^{5,10}$-methenyl derivative in 0.25 M HCl. The results were in good agreement with those determined by HPLC (pH 3, 37 ± 6%; pH 5, 85 ± 6%; pH 7, 12 ± 3%). The lack of detectable synthesis of the

Table 2

Yields of products of the reaction of tetrahydrofolate with formyl phosphate[a]

pD	Formate[b]	2-Mercapto-ethylformate[b]	N^5-formyl-THF[c]
3	ND[d]	ND[d]	45
5	51 ± 10	29 ± 4	64
7	84 ± 10	6 ± 4	8

[a] The data are from a 24-h reaction as described in the text. Results are presented as percent of formyl phosphate for formate, percent of 2-mercaptoethanol for 2-mercaptoethylformate and percent of THF for N^5-formylTHF

[b] Determined from ^{1}H-NMR spectrum

[c] Determined from HPLC analysis

[d] ND = not determined

N^{10}-formyl derivative was not altered by the inclusion of $MgCl_2$ to 0.1 M, 50% dimethylsulfoxide or 50% acetonitrile.

Previous results demonstrated that formyl phosphate formylates primary amines [9] and nitrogen containing macrocycles and linear polyamines [10,11] under mild conditions. The results presented in this communication show that although formyl phosphate also formylates THF, it does so exclusively at the N^5 position, within the detection limits of our analytical methods. Under the conditions used we found no evidence for the formation of the N^{10}-formyl derivative, nor did we find evidence for the formation of a phosphoramide, either as an intermediate or a final product. In this respect the reaction is similar to that of formyl phosphate with nitrogen-containing macrocycles; bond cleavage is exclusively at the C-O bond [10]. The reaction of formyl phosphate with THF was pH-dependent with a higher yield occurring at pH 5 than at pH 7 or pH 3. Since the competing hydrolysis rate does not vary between pH 5 and 7 [10] we surmise that the increase at pH 5 is due to the protonation of the formyl phosphate to give the monoanionic species. The decrease in yield at pH 3 could be due to a more rapid loss of formyl phosphate by way of hydrolysis [9] and/or to the protonation of the 5 nitrogen [12].

N^5-FormylTHF has been synthesized from THF and methyl formate in dimethylsulfoxide [13], and formic acid with a carbodiimide in an aqueous solution [14]. In these reactions the N^{10} derivative also was not produced. Because of the electron withdrawing effect of the aromatic ring in the *p*-aminobenzoic moiety, a lower reactivity of the 10 nitrogen compared to the 5 nitrogen would be expected. N^{10}-Formyltetrahydrofolate synthetase, therefore, completely changes the regioselectivity of the reaction between THF and formyl phosphate. This suggests that the enzyme, in addition to enhancing the reactivity of the 10 nitrogen, must also decrease the reactivity or accessibility of the 5 nitrogen. Because N^5-methylTHF and N^5-formylTHF

apparently do not bind to the enzyme, as judged by their lack of inhibitory activity [15], it would appear that the 5 nitrogen is hindered from accepting a formyl group at the active site.

Acknowledgements: This work was supported by NIH Grant DK 07140 and in part by a grant from the University of Kansas Biomedical Sciences Support Fund.

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