Biochimica et Biophysica Acta, 613 (1980) 73—78

BBA 68944

INHIBITOR STUDIES OF METHIONYL-tRNA TRANSFORMYLASE OF *EUGLENA GRACILIS*

ANNASTELLA GAMBINI, PAOLO CROSTI and RENATO BIANCHETTI

Centro di Studio del Consiglio Nazionale delle Ricerche per la Biologia Cellulare e Molecolare delle Piante, Istituto di Scienze Botaniche, Università di Milano, Via G. Colombo 60, 20133 Milano (Italy)

(Received August 15th, 1979)

Key words: 10-Formyltetrahydrofolate:Met-tRNA^f transformylase; Inhibitor; Folate metabolism; Pteroylglutamate analog; (Euglena gracilis)

Summary

Inhibitor studies of the only known eukaryotic methionyl-tRNA transformylase (10-formyltetrahydrofolate:L-methionyl-tRNA *N*-transformylase, EC 2.1.2.9) were carried out. All the natural pteroylglutamic acid derivatives examined, with the exception of pteroylglutamic acid, are inhibitors. The most effective is 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) ($K_I = 3 \cdot 10^{-6}$ M), which is the only noncompetitive inhibitor of the enzyme. All the other derivatives tested are competitive, and H_4PteGlu shows a cooperative inhibition. These and other data obtained with pteroylglutamic analogues show that, in contrast to the bacterial enzyme, *Euglena* transformylase is also inhibited by compounds without a fully reduced pyrazine ring and is very sensitive to compounds with a methyl group in position 5 or 10 of the pteridine ring.

Introduction

Methionyl-tRNA transformylase (10-formyltetrahydrofolate:L-methionyl-tRNA *N*-transformylase, EC 2.1.2.9) is the enzyme that catalyzes the transfer of the formyl group from 10-formyltetrahydrofolate (10-CHO-H_4PteGlu) to the amino group of Met-tRNA^f. It is the tRNA specifically required for the initiation of protein synthesis in prokaryotes, as well as in mitochondria and chloroplasts. The formylation reaction catalyzed by the transformylase is generally considered as an absolute requirement to initiate peptide chains in bacterial [1], mitochondrial and chloroplast protein-synthesis systems [2,3]. Inhibitor studies of the bacterial enzyme, but not of the organelle enzyme,

have already been published [4]. The enzyme from *Euglena gracilis* chloroplast is, at present, the only eukaryotic transformylase extensively purified and well characterized. This enzyme shows many different properties to those of the bacterial enzyme, the most interesting of which is a strong positive cooperativity for both the substrates [5].

In this study, the inhibitory effects of the most important natural pteroylglutamic acid derivatives and some pteroylglutamic acid analogues on *E. gracilis* transformylase were examined.

Materials and Methods

Cultures of *E. gracilis* were maintained axenically in Hutner's medium. Chloroplast transformylase purification, transformylase assay, and 10-[^{14}C]HO-H_4PteGlu and [^{3}H]methionyl-tRNA^f preparations have been described previously [5]. Tetrahydrofolate (H_4PteGlu), 5-methyl-tetrahydrofolate (5-CH_3-H_4PteGlu) and pteroylglutamic analogues (aminopterin and methotrexate) are from Sigma. 5-Formyltetrahydrofolate (5-CHO-H_4PteGlu) is a commercial product and was further purified by use of a cellulose column [6]. 5,10-Methylene-tetrahydrofolate (5,10-CH_2-H_4PteGlu) was prepared according to Ramasastri and Blakely [7] to obtain it free of H_4PteGlu. H_4-aminopterin was prepared from aminopterin by catalytic hydrogenation in glacial acetic acid [8]. Formylpteroylglutamic acid and formylaminopterin were obtained according to the method of Mathews and Huennekens [9]. All the compounds used were routinely checked by their absorption spectra.

Results

Pteroylglutamic acid derivatives

10-CHO-H_4PteGlu, the formyl group donor in the transformylase reaction, is an important compound on the H_4PteGlu pathway. Pteroylglutamic acid, H_4PteGlu itself, an isomeric form of 10-CHO-H_4PteGlu, 5-CHO-H_4PteGlu, 5-10-CH_2-H_4PteGlu and 5-CH_3-H_4PteGlu are the natural compounds whose inhibitory effect on the transformylase was tested. All these compounds, with the exception of pteroylglutamic acid, showed a powerful inhibitory action on the transformylase. However, they appeared to differ greatly in the mechanism of inhibition. 5-CHO-H_4PteGlu is competitive, 5-CH_3H_4PteGlu is noncompetitive (Fig. 1) and H_4PteGlu is a competitive inhibitor which shows cooperation in the binding. All the data obtained and the kinetic methods [10] are summarized in Table I.

Since the inhibition by H_4PteGlu had an unusual behaviour, its detailed kinetic analysis is reported. First, the inhibition by H_4PteGlu was analyzed by constructing the Dixon plot ($1/v$ vs. [H_4PteGlu]) in the presence of two different concentrations of 10-CHO-H_4PteGlu. The plot obtained was curved and did not allow an identification of the type of inhibition. Since the substrate binds cooperatively, the Hill equation for multisite inhibition:

$$\log \frac{v_i}{v_0 - v_i} = -n \cdot \log [I] + \log K'$$

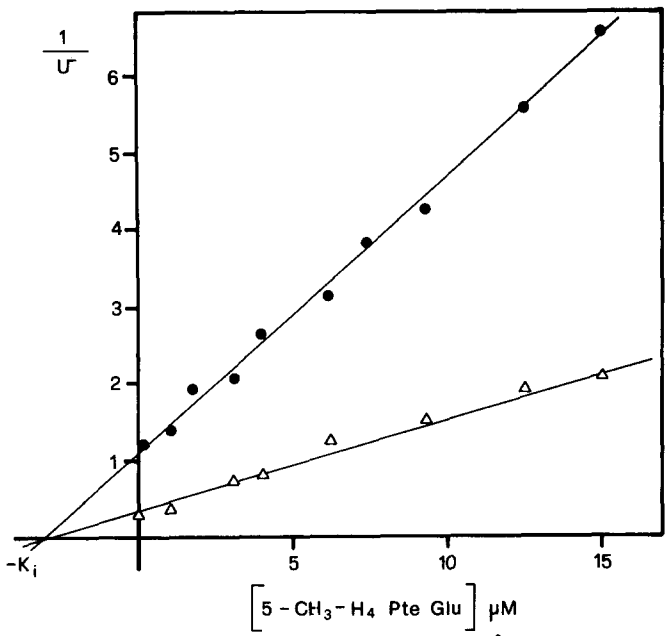

Fig. 1. Dixon plot of inhibition of the Met-tRNA^f transformylase activity by 5-CH_3-H_4-PteGlu. △, 10-CHO-H_4PteGlu, 0.76 μM; ●, 10-CHO-H_4PteGlu, 0.35 μM.

was tested and the Hill plot was constructed. Straight lines with a slope (n_{app}) of 1.64 were obtained (Fig. 2). The Dixon plot obtained by raising to a power of 1.64 the [I] (H_4PteGlu concentration) yielded straight lines that intersected above the [I]-axis, and a K_I' of $0.2 \cdot 10^{-6}$ M was measured from the graph. This value of K_I' is very close to that calculated from the $[I]_{50}$ by the relation $K_I' = [I]_{50}^n$. The data obtained were also analyzed by the Lineweaver-Burk plot. The family of reciprocal plots intersect above the 1/[S]-axis, (S, substrate) to the

TABLE I

EFFECT OF FOLATE DERIVATIVES ON Met-tRNA^f TRANSFORMYLASE ACTIVITY

The type of inhibition and K_I values were calculated from a Dixon plot ($1/v$ vs. [I]); the concentrations of 10-CHO-H_4PteGlu were 0.35 and 0.76 μM, respectively. K_I' was calculated from a linearized Dixon plot (see text). No attempt to separate isomers was made in these inhibitory studies. The inhibition constant values were calculated from the total concentration of the compounds. A compound was evaluated as noninhibitory if it caused less than a 15% inhibition at the concentration of 1 mM under standard conditions. n_{app} was calculated from a Hill plot. $[I]_{50}$ was calculated plotting v_i/v vs. [I]. The concentration of 10-CHO-H_4PteGlu was 0.6 μM, corresponding to $[S]_{0.5}$.

Compound	Inhibition type	n_{app}	K_I or K_I' ($\times 10^{-6}$) (M)	$[I]_{50} \times 10^{-6}$ (M)
Pteroylglutamic acid	noninhibitory	—	—	—
H_4PteGlu	competitive	1.64	$K_I' = 0.2$	60
5-CHO-H_4PteGlu	competitive	1.06 *	$K_I = 45$	90
5-10-CH_2-H_4PteGlu	—	—	—	20
5-CH_3-H_4PteGlu	noncompetitive	—	$K_I = 3$	3

* The straight line of the Hill plot was calculated on the basis of linear regression, the value of 1.06 was considered equal to 1.

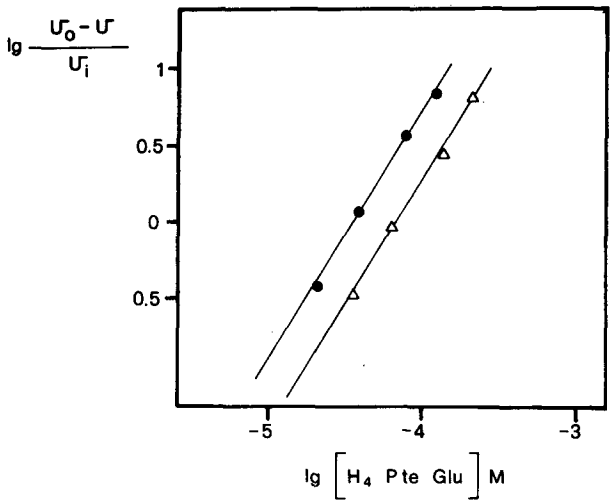

Fig. 2. Inhibition of Met-tRNA^f transformylase activity by H_4PteGlu: Hill plot. △, 10-CHO-H_4PteGlu, 1.15 μM; ●, 10-CHO-H_4PteGlu, 0.57 μM. The straight lines of the Hill plot were calculated on the basis of linear regression.

right of the $1/v$-axis; the slope replot is curved, and the Hill plot of this replot against inhibitor concentration yields an n_{app} of 1.7. All these results are in accordance with a competitive, multisite inhibition. In multisite inhibition, the inhibitors can yield n_{app} values greater than 1.0 in the absence of true cooperative binding [11]. However, the n_{app} obtained, calculated from experiments carried out at low inhibitor concentration, is probably high enough to support the conclusion of a two-site system with cooperative binding of the inhibitor. No evidence of a similar behaviour was obtained for the other inhibitors tested.

Pteroylglutamic acid analogues

Some of the most important inhibitors of H_2PteGlu reductase were tested as inhibitors of the transformylase, and they are listed in Table II according to their effectiveness. Trimethoprim, which does not possess the pteridine ring,

TABLE II

INHIBITION OF Met-tRNA^f TRANSFORMYLASE ACTIVITY BY PTEROYLGLUTAMIC ACID ANALOGUES

A compound was evaluated as noninhibitory (n.i.) if it caused less than a 15% inhibition at the concentration of 1 mM under standard assay conditions. Values are presented as percentage inhibition.

PteGlu analogue	Concentration of the inhibitor (mM)		
	0.01	0.1	1.0
Trimethoprim	n.i.	n.i.	n.i.
Aminopterin	—	22	26
Reduced aminopterin	—	28	34
Formylaminopterin	38	62	—
Methotrexate	30	52	—
Formyl-PteGlu acid	—	31	52

has no inhibitory effect. Aminopterin (4-amino 4-deoxyfolic acid) is only a weak inhibitor; its reduced form (4-amino 4-deoxy H_4folic acid) is more efficacious but less than expected from the difference in inhibition between pteroylglutamic acid (noninhibitory) and H_4PteGlu ($[I]_{50} = 6 \cdot 10^{-5}$ M). Both these analogues showed behaviours typical of the partial competitive inhibitors, but this aspect was not sufficiently analyzed. The introduction of a formyl group in position 10 of the pteroylglutamic acid (10-CHO-PteGlu) brings about a remarkable inhibitory action, and the same occurs when aminopterin is formylated (10-CHO-aminopterin). A high level of inhibition remains even if the formyl group of the 10-CHO-aminopterin is substituted by a methyl group (methotrexate).

All the experiments reported here were carried out utilizing a highly-purified enzyme preparation. However, the gel electrophoresis of this preparation shows two bands: as judged by molecular weight previously measured by gel filtration, the major band clearly corresponds to 10-CHO-H_4PteGlu:Met-tRNA^f transformylase and the minor band could correspond to a species of enzyme with a different molecular weight. Actually, we did not obtain direct evidence for the enzymatic activity of the latter band. In addition, it represents less than 5% of the major band and will not be considered in the discussion [5].

Discussion

Starting from pteroylglutamic acid, the structural modifications in the pteridine derivatives studied as inhibitors are substantially the following: (1) the reduction in positions 5, 6, 7 and 8 of the pteridine ring (pyrazine ring), (2) the substitution of the hydroxyl group in position 4 with an NH_2 group (aminopterin and its derivatives), (3) the introduction in positions 5 or 10 of a formyl or methyl group. All these modifications can increase the inhibitory effect, but the results are not cumulative. In addition, 1 and 3 increase the structural similarities to the substrate, but 2 does not. Thus, in contrast to the *Escherichia coli* enzyme [4], the attempt to define for the chloroplast enzyme the contribution to the binding of the various parts of the substrate molecule seems largely premature. However, a brief comparison with the bacterial enzyme seems to be useful. The most striking differences are: (1) there is no absolute requirement for a fully-reduced pyrazine ring — in fact, aminopterin, formylaminopterin, formylpteroylglutamic acid and methotrexate are inhibitors; (2) a methyl group at nitrogen position 5 or 10 is more effective on the chloroplast than on the bacterial enzyme. With respect to H_4PteGlu, the 5-CH_3 derivative has a greater inhibitory effect on the chloroplast and a lesser effect on the bacterial enzyme. In addition, L-10-CH_3PteGlu does not inhibit the bacterial enzyme, whereas methotrexate is a very strong inhibitor of the chloroplast enzyme. On the contrary, it might be relevant to note that *Euglena* H_2PteGlu reductase is much less inhibited than the bacterial enzyme by aminopterin and methotrexate [12].

It could be useful to define the significance of the inhibition of the organelle transformylase by the naturally-occurring folate derivatives. The absolute requirement for the formylation of the initiator, Met-tRNA^f, in both chloroplasts and mitochondria [2,3], establishes a strong link between the folate-

mediated macromolecular biosynthesis and the initiation of peptide chains in the organelles. At least a part of the coordinative aspects of these biosyntheses could be realized through a concerted action of the H_4PteGlu derivatives on the transformylase.

At the moment, any evaluation of this hypothesis is difficult, since unusual difficulties are encountered in the study of the mitochondrial enzyme (we tried yeast and rat liver enzymes), and there is very little information about the presence and the function of the H_4PteGlu-related coenzymes in the chloroplast [13,14]. For example, the noncompetitive inhibition of the chloroplast enzyme by 5-CH_3-H_4-PteGlu with a very low K_I ($3 \cdot 10^{-6}$ M) could be very interesting, since 5-CH_3-H_4PteGlu is the predominant form of the H_4PteGlu coenzymes, and its concentration can widely vary in the cell. However, at the moment, there is no evidence for the presence of this compound in the chloroplast. In addition, it should be noted that the naturally occurring folates are a mixture of mono- and polyglutamyl forms; the latter ones might have a degree of inhibition very different to that of the forms studied here.

A regulative role on the Met-tRNA^f transformylase activity could be ascribed to the H_4PteGlu, which is a product of the reaction and the only compound that shows a cooperative inhibition. Clearly the ratio between H_4PteGlu alone and H_4PteGlu with its C_1 adduct (formyl, methylene, methyl group) depends on the velocity ratio between several catabolic and anabolic pathways. High levels of H_4PteGlu might correspond to limiting amounts of C_1-transferring-H_4PteGlu forms, and the inhibition of Met-tRNA^f transformylase could be useful to regulate the conversion to more reduced forms of the H_4PteGlu-linked formyl groups.

References

1 Lucas-Lenard, J. and Lipmann, F. (1971) Annu. Rev. Biochem. 40, 409—448
2 Bianchetti, R., Lucchini, G., Crosti, P. and Tortora, P. (1977) J. Biol. Chem. 252, 2519—2523
3 Lucchini, G. and Bianchetti, R. (1980) Biochim. Biophys. Acta, in the press
4 Dickerman, H.W. and Smith, B.C. (1970) Biochemistry 9, 1247—1255
5 Crosti, P., Gambini, A., Lucchini, G. and Bianchetti, R. (1977) Biochim. Biophys. Acta 477, 356—370
6 Huennekens, F.M., Ho, P.P.K. and Scrimgeour, K.G. (1963) Methods Enzymol. 6, 810
7 Ramasastri, B.V. and Blakly, R.L. (1962) J. Biol. Chem. 237, 1982—1988
8 Kisliuk, R.L. (1957) J. Biol. Chem. 277, 805—814
9 Mathews, C.K. and Huennekens, F.M. (1963) J. Biol. Chem. 238, 3436—3442
10 Segel, I.H. (1975) Enzyme Kinetics, pp. 100—160, Wiley-Interscience Publication, J. Wiley and Sons, New York
11 Segel, I.H. (1975) Enzyme Kinetics, pp. 470—473, Wiley-Interscience Publication, J. Wiley and Sons, New York
12 Bianchetti, R. and Crosti, P. (1979) Acad. Naz. Lincei Rend. Sci. Fis. Mat. Nat. 66, 57—61
13 Shad, S.P.J. and Cossins, E. (1970) Phytochemistry 9, 1545—1551
14 Crosti, P. (1974) Ital. J. Biochem. 23, 72—86