Biochimica et Biophysica Acta, 524 (1978) 55—59

BBA 68430

SERINE TRANSHYDROXYMETHYLASE

EQUILIBRIUM BINDING OF FOLATE ANALOGS AS ACTIVE SITE PROBES

C.W. JONES, III, J.B. HYNES and D.G. PRIEST

Department of Biochemistry, Medical University of South Carolina, Charleston, S.C. 29403 (U.S.A.)

(Received September 6th, 1977)

Summary

Formation of a quinoid-like structure within the glycyl-pyridoxal phosphate moiety of serine transhydroxymethylase (5,10-methylenetetrahydrofolate : glycine hydroxymethyltransferase, EC 2.1.2.1) is dependent upon the dissociation of the 2-S hydrogen of glycine which in turn requires the presence of tetrahydrofolate or analogs thereof. Equilibrium binding studies with the series folate, dihydrofolate, and tetrahydrofolate showed that reduction of the pteridine ring enhances both quinoid formation and binding. A 5,8-deazafolate series showed that modifications in the 4 position, 10 position and the glutamyl position yield interrelated alterations of quinoid formation which could not be correlated with binding.

Introduction

Schirch and Ropp [1] have observed an absorption band at 495 nm due to the formation of a ternary complex between rabbit liver serine transhydroxymethylase (5,10-methylenetetrahydrofolate : glycine hydroxymethyltransferase, EC 2.1.2.1) glycine and tetrahydrofolate. This band has been attributed to a quinoid-type structure within the glycyl-pyridoxal phosphate moiety of the enzyme active site [2,3]. A series of folate analogs have been used to systematically probe the active site of beef liver serine tranhydroxymethylase, both with regard to formation of the quinoid structure and requirements for binding. These results, in combination with previous investigations [4], have been interpreted in terms of a proposed mechanism of action.

Materials and Methods

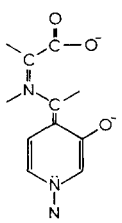
Folate was obtained from Sigma Chemical Company and methotrexate from Nutritional Biochemicals Corporation. Dihydrofolate was prepared by the

dithionite method of Futterman [5] as modified by Blakely [6]. Tetrahydrofolate was prepared as previously described [4]. Compounds 12, 13, 14, and 15 in Table I were prepared as described by Hynes et al. [7], compounds 6 and 11 by Acharya and Hynes [8], compounds 7 and 8 by Hynes and Garrett [9], and compounds 5, 9 and 10 by Hynes et al. [10].

Beef liver serine transhydroxymethylase was purified as described by Schirch and Gross [11] and Schirch and Mason [12] for the rabbit liver enzyme or using the affinity chromatographic method previously described [4]. Specific activity was determined by the β-phenylserine aldolase assay [4], and no enzyme preparation with a specific activity of less than 2.0 units/mg protein was used. A Beckman Acta CIII ultraviolet-visible spectrophotometer with cell chamber maintained thermostatically at 25°C was used throughout.

Results

Complexes formed between beef liver serine transhydroxymethylase, glycine and tetrahydrofolate absorb at 495 nm [1]. No detectable shift in absorption maximum was observed when tetrahydrofolate was replaced by other folate derivatives used in this study. The relative magnitude of the absorption band varies greatly. If it is assumed that this band can be attributed to the formation of the same quinoid-like structure in enzyme bound glycyl-pyridoxal phosphate as that shown below [2,3]



absorbance per unit enzyme reflects the relative concentration of this species. Extrapolation to saturating conditions of both glycine and each folate derivative allowed estimation of apparent molar extinction coefficients, ϵ_{495}, for each complex (Table I). Under these conditions, the magnitude of the apparent extinction coefficient is a direct indication of the equilibrium concentration of the quinoid-like species and hence the ability of each derivative to promote formation of this species.

Absorbance as a function of both glycine and folate derivative concentration provides a basis for the estimation of dissociation constants for each of the elementary steps in the binding cycle leading to the formation of a ternary complex. The elementary steps and related binding constants are shown below,

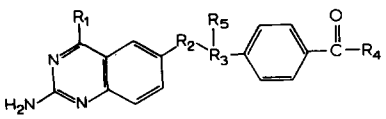
$$E + G \overset{K_G}{\rightleftharpoons} EG \qquad EL + G \overset{K'_G}{\rightleftharpoons} ELG$$

$$E + L \overset{K_L}{\rightleftharpoons} EL \qquad EG + L \overset{K'_L}{\rightleftharpoons} ELG$$

where G is glycine, L is folate derivative and E is serine transhydroxymethylase.

TABLE I

TERNARY COMPLEX ABSORPTIVITY AND DISSOCIATION CONSTANTS FOR GLYCINE/FOLATE ANALOG BINDING CYCLE



Compound No.	ϵ_{495}	R_1	$-R_2\ R_3-$	R_4	R_5	$K_L \times 10^5$	$K'_L \times 10^5$	$K_G \times 10^5$	$K'_G \times 10^5$
1	5 000		Folate			8.3	7.7	800	590
2	19 000		Dihydrofolate			6.6	5.6	800	1100
3	44 000		Tetrahydrofolate			5.7	1.4	800	160
4	3 100		Methotrexate			28	11	800	430
5	69 000	$-NH_2$	$-CH_2NH-$	—Glu	—H	8.3	7.8	1060	540
6	4 200	—OH	$-CH_2NH-$	—Glu	—H	11	9.5	820	740
7	25 000	$-NH_2$	$-NHCH_2-$	—Glu	—H	50	14	870	460
8	9 400	—OH	$-NHCH_2-$	—Glu	—H	18	67	730	890
9	23 300	$-NH_2$	$-CH_2NH-$	—Glu	—CHO	17	28	750	500
10	12 100	—OH	$-CH_2NH-$	—Glu	—CHO	28	13	680	580
11	9 300	—OH	$-CH_2NH-$	—Glu	$-CH_3$	20	8.7	830	400
12	12 100	$-NH_2$	$-CH_2NH-$	—OEt	—H	50	11	690	330
13	19 000	—OH	$-CH_2NH-$	—OEt	—H	100	12	850	250
14	16 700	$-NH_2$	$-NHCH_2-$	—OEt	—H	25	25	910	480
15	16 800	—OH	$-NHCH_2-$	—OEt	—H	12	12	830	350

Discussion

Chen and Schirch [12] have observed a dramatic increase in the rate of serine transhydroxymethylase catalysed exchange of [2*S*-^{3}H]glycine with water protons upon addition of tetrahydrofolate. This increased exchange rate is consistant with the formation of a glycylimine, which in turn gives rise to the 495 nm absorbing quinoid structure in the pyridoxal phosphate ring. Although the participation of this enzyme species during catalysis has not been directly proven, its presence along the reaction pathway leading to cleavage of the β-hydroxymethyl function of serine and its transfer to reduced folate is strongly implicated [2]. The ability of folate derivatives to generate this 495 nm absorbing species or quinoid-like structure under conditions that do not allow transfer of the hydroxylated carbon can give insight into the specific manner in which the chromaphore is formed.

Folate derivatives (and analogs) with configurations which enhance removal of the 2*S*-hydrogen from glycine also enhance formation of the quinoid-like chromaphore. The first 3 compounds in Table I, folate, dihydrofolate, and tetrahydrofolate, have increasing abilities to enhance formation of the quinoid. This increased hydrogen withdrawal can be correlated with increased basicity of the N^5-nitrogen in the pteridine ring. Such an interpretation is also consistent with the relatively poor enhancement observed with methotrexate, in which the ring is fully oxidized. The further appeal of placing the N^5-nitrogen close to the 2-carbon of glycine is to put it into proximity for direct acceptance

of the hydroxymethyl group by tetrahydrofolate from serine during the serine transhydroxymethylase reaction.

It would be an oversimplification, however, to expect basicity of N^5 to completely predict formation of quinoid-like structure in this complex system. That this is not the case can easily be seen by reference to compound 5 in Table I; a 5,8-deazafolate in which the 4-hydroxyl is replaced by a 4-amino function. Obviously, there is no N^5 at all in this oxidized quinazoline ring system, but the quantity of quinoid structure formed is higher than with any other compound tested, even tetrahydrofolate. The superiority of the 4-amino over the 4-hydroxy derivative is generally adhered to throughout the 5,8-deazafolate series, suggesting significant contributions from functional groups in this position.

In addition to N^5, the N^{10} position might also logically be expected to be located near the 2-carbon of glycine since it is the other point of bonding for the methylene groups in methylene tetrahydrofolate, one of the products of the serine transhydroxymethylase reaction. Methotrexate, compound 4, in which the N^{10} position is occupied by a methyl group, is a poor generator of quinoid-like structure. However, comparison of methotrexate with tetrahydrofolate is not entirely satisfactory because of substitution of an amino function at the 4 position and full oxidation of the pteridine ring. Comparison of methotrexate with compound 5 is also hampered by the lack of a 5,8-deaza configuration. Comparisons within the 5,8-deaza series are more useful.

The following approaches to the examination of the influence of the N^{10} position within the 5,8-deaza series have been taken: substitution of formyl or methyl groups at N^{10} (compounds 9, 10 and 11) and inversion of the normal $-CH_2NH-$ bridge to the isofolate configuration $-HNCH_2-$ (compounds 7, 8, 14 and 15). Compound 9, the N^{10}-formyl derivative of compound 5, was only about one-third as capable of quinoid generation as the parent compound, 5. Compounds 10 and 11, N^{10}-formyl and N^{10}-methyl derivatives of compound 6, showed better quinoid promotion than their 4-hydroxy parent compound, 6. Thus, while substitution of amino function in the 4 position appears to dominate, its effect can be diminished by chemical alterations at N^{10}. Examination of the inverted bridge series supports this conclusion. Compound 7, the inverted bridge cogener of compound 5, is less capable of quinoid formation but again the reverse relationship is seen when the 4-hydroxy compounds (6 and 8) are compared.

The series in which glutamate is replaced by an ethyl ester (compounds 12—15) allows evaluation of the effects of this substituent in terms of the relative ability to alter effects of substituents at the 4 carbon and N^{10} positions. It can be seen that without glutamate, the enhancement caused by 4-amino substitution is reversed (compounds 12 and 13), while bridge reversal has little or no impact. This suggests that at least one of the free carboxyl functions of glutamate can occupy a region sufficiently close to the 2-carbon of glycine in the active site to perturb the effects caused by substitution at the 4-carbon and N^{10} positions.

Glycine, required in the ternary complex for quinoid formation, also affects the dissociation constants for folate and folate derivative binding [1,4]. It can be seen that in the folate series (compounds 1—3 of Table I) reduction of the

pteridine ring results in enhanced binding to the enzyme-glycine complex, K'_L, but has little effect upon binding to the free enzyme, K_L. Again, as was true with quinoid formation, the correlation is not so simple when extended to the 5,8-deazafolate series. Binding enhancement due to the presence of glycine does occur in some cases, but in contrast to the folate series there is no apparent direct correlation between quinoid formation and binding to either form of the enzyme.

Binding of glycine is also different in the presence (K'_G) and absence (K_G) of folate derivative. Values of K_G are self consistent and in good agreement with those previously reported [1]. Binding enhancement of glycine was most pronounced with tetrahydrofolate but was significant for several of the folate derivatives studied.

Enhancement of β-phenylserine binding by folate, tetrahydrofolate and a 5,8-deazafolate (compound 13) has been proposed as the mechanism to explain rate enhancement for the β-phenylserine cleavage reaction catalyzed by beef liver serine transhydroxymethylase [4]. An alternative mechanism which has not previously been ruled out is folate derivative influence upon the energetics of the transition state (i.e. an effect upon catalytic reactivity rather than binding). Such a mechanism would presumably be mediated through interaction of the folate derivative with the amino acid-pyridoxal phosphate moiety within the active site. Such an interaction should affect the amount of quinoid-like structure formed. Tetrahydrofolate and folate activate with regard to β-phenylserine cleavage to about the same extent, whereas the 5,8-deazafolate (compound 13) is only approximately one-half as effective [4]. However, tetrahydrofolate is many times more effective than folate as a quinoid generator, and the 5,8-deaza derivative is intermediate. Thus either the quinoid is of little importance to the specific catalytic event, or the major influence of folate and folate derivatives in the facilitation of β-phenylserine cleavage is upon enhancement of substrate binding. While the latter appears most likely, further studies to ascertain the precise role of the quinoid-like structure in catalysis will be needed to unequivocally resolve this question.

Acknowledgements

This work was supported by the South Carolina Appropriation for Research.

References

1 Schirch, L. and Ropp, M. (1967) Biochemistry 6, 253—257
2 Schirch, L. (1975) J. Biol. Chem. 250, 1939—1945
3 Schirch, L. and Jenkins, W.T. (1964) J. Biol. Chem. 239, 3801—3807
4 Jones, C.W. III, and Priest, D.G. (1976) Arch. Biochem. Biophys. 174, 305—311
5 Futterman, S. (1957) J. Biol. Chem. 228, 1031—1038
6 Blakley, R.L. (1960) Nature 188, 231—232
7 Hynes, J.B., Buck, J.M., D'Souza, L. and Freisheim, J.H. (1975) J. Med. Chem. 18, 1191—1194
8 Acharya, S., Prakash, C. and Hynes, J.B. (1975) J. Heterocyclic Chem. 12, 1283—1286
9 Hynes, J.B. and Garrett, C.M. (1975) J. Med. Chem. 18, 632—634
10 Hynes, J.B., Eason, D.E., Garrett, C.M., Colvin, P.L., Shores, K.E. and Freisheim, J.H. (1977) J. Med. Chem. 20, 588—591
11 Schirch, L. and Gross, T. (1968) J. Biol. Chem. 243, 5651—5655
12 Schirch, L. and Mason, M. (1963) J. Biol. Chem. 238, 1032—1037