Thermodynamic Analysis of the Binding of the Polyglutamate Chain of 5-Formyltetrahydropteroylpolyglutamates to Serine Hydroxymethyltransferase†

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ABSTRACT: The thermodynamic parameters for the binding of 5-formyltetrahydrofolate (5-CHO-H_4PteGlu$_n$) and its polyglutamate forms to rabbit liver cytosolic serine hydroxymethyltransferase (SHMT) were determined by a combination of isothermal titration calorimetry and spectrophotometry. Binding of 5-CHO-H_4PteGlu$_n$ to SHMT exhibits both positive enthalpy and entropy, showing that binding is entropically driven. 5-CHO-H_4PteGlu$_5$ has a 300-fold increased affinity for SHMT compared to 5-CHO-H_4PteGlu. This increase in affinity is due primarily to a decrease in the positive enthalpy with little change in entropy. A variety of anions inhibit the binding of 5-CHO-H_4PteGlu$_5$ with K_i values in the 10−20 mM range. Anions are ineffective inhibitors of 5-CHO-H_4PteGlu binding to SHMT, showing that anions compete for the polyglutamate binding site. There was little difference in the K_i values for a series of dicarboxylic acids as inhibitors of 5-CHO-H_4PteGlu$_5$, suggesting that spacing of the negative charges may not be important in determining their effectiveness as inhibitors. Both the mono- and pentaglutamate derivatives of 5-CHO-H_4PteGlu$_n$ were cross-linked to SHMT by a carbodiimide reaction to Lys-450 which resides in a stretch of Lys, His, and Arg residues.

Tetrahydrofolate (H_4PteGlu$_n$)[1] functions as a coenzyme in cellular metabolism as a carrier of one-carbon groups. Enzymes that require the one-carbon derivatives of tetrahydrofolate participate in de novo purine, methionine, and thymidylate biosynthesis. In addition, a derivative of this coenzyme is the direct source of the methyl group of AdoMet that is used for methylating many compounds in the cell, including DNA and RNA. The physiologically active form of H_4PteGlu$_n$ requires the addition of glutamate residues which are linked as amides through the γ-carboxyl group. Even though n is 5−7 for H_4PteGlu$_n$ in most cells, it has recently been shown with Chinese hamster ovary cells that n may only need to be 3 to meet the physiological requirement of the cell (*1*).

Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) catalyzes the transfer of the 3-hydroxymethyl group of serine to H_4PteGlu$_n$ to form CH_2-H_4PteGlu$_n$ and glycine. Like many other H_4PteGlu$_n$-requiring enzymes, SHMT exhibits a significant increase in affinity for H_4PteGlu$_n$ as the number of glutamate residues increases (*2*). Determining the thermodynamic parameter responsible for the increased affinity for the active site requires measurement of the change in enthalpy for H_4PteGlu$_n$ binding. This has been difficult because of the instability of the derivatives of H_4PteGlu$_n$. To our knowledge, the role of enthalpy and entropy in the binding process of a polyglutamate form of H_4PteGlu$_n$ has not been determined for any folate-requiring enzyme.

5-CHO-H_4PteGlu$_n$ is the only stable derivative of H_4-PteGlu$_n$. We have shown previously that this derivative binds tightly to SHMT with affinities similar to that of H_4-PteGlu$_n$ as n increases from 1 to 5 (*3*). The binding can be conveniently monitored by a spectrophotometric method involving an abortive ternary complex with glycine (*4*). Using both isothermal titration calorimetry and this spectrophotometric method, we have approached the problem of defining the thermodynamic parameters involved in H_4PteGlu$_n$ binding. We address the questions about how the polyglutamate chain of 5-CHO-H_4PteGlu$_n$ increases the affinity for the active site and the residues involved in binding.

EXPERIMENTAL PROCEDURES

Materials. Pteroylpolyglutamates and (6*S*)-5-CHO-H_4-PteGlu were purchased from B. Schircks (Jona, Switzerland). *Lactobacillus casei* dihydrofolate reductase was a generous gift from R. Kisliuk (Tufts University, Boston, MA). 5-^{14}CHO-H_4PteGlu$_5$ (9 mCi/mmol) was synthesized according to a method described by Stover and Schirch (*5*). The di-, tri-, tetra-, and pentaglutamate forms of (6*S*)-5-CHO-H_4PteGlu$_n$ were synthesized starting from the corresponding PteGlu$_n$ (*5*). (6*RS*)-[3′,5′,7,9-^{3}H]leucovorin (21 Ci/mmol) was purchased from Moravek Biochemicals, Inc., and [^{14}C]-formate (55 mCi/mmol) was purchased from Amersham. 5,10-Methenyltetrahydrofolate synthetase was purified from

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[1] Abbreviations: SHMT, serine hydroxymethyltransferase; 5-CHO-H_4PteGlu$_n$, 5-formyltetrahydrofolate containing n glutamate residues; 5-CH_3-H_4PteGlu and H_4PteGlu, 5-methyltetrahydrofolate and tetrahydrofolate, respectively.

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rabbit livers (*6*). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide and other reagents were purchased from Sigma.

SHMTs. Rabbit liver cytosolic serine hydroxymethyltransferase was purified from fresh frozen livers obtained from Pel-Freeze Biological (Rogers, AK) according to the protocol described previously (*5*). The protein concentration was determined from its ϵ_{278nm} of 38 900 M^{-1} cm^{-1} (*7*). *Neurospora crassa* SHMT and recombinant human liver cytosolic SHMT were purified as described previously and their concentrations determined using extinction coefficients of 37 500 and 33 400 M^{-1} cm^{-1}, respectively (*8*, *9*).

UV Spectrophotometry. Binding of 5-CHO-H_4PteGlu$_n$ by SHMT in the presence of 50 mM glycine was monitored at 502 nm on a Hewlett-Packard 8452A diode array spectrophotometer. The experiments were performed at 25 °C in 20 mM potassium phosphate buffer (pH 7.0). Concentrations of SHMT near the corresponding K_d values of 5-CHO-H_4-PteGlu$_n$ (0.1−10 μM) were titrated with microliter aliquots of 5-CHO-H_4PteGlu$_n$ of mono-, di-, tri-, tetra-, and pentaglutamate, respectively, until the absorbance at 502 nm reached its maximum absorbance. The 5-CHO-H_4PteGlu$_n$ solutions were made in high concentrations so that the volume of the total added ligand was less than 5% of the final volume in the cuvette. After each addition of ligand, the solution was incubated for 5 min prior to recording the absorbance at 502 nm. 5-CHO-H_4PteGlu$_n$ titration experiments were performed in a 1 cm cuvette, and all the other titrations of longer glutamate derivatives were performed in a 10 cm cuvette. The titrations of 5-CHO-H_4PteGlu$_n$ performed at other temperatures were carried out in either a water bath or by placing the entire spectrophotometer in a room at the desired temperature.

Determination of K_d and K_i Values. The absorbance data described in the previous paragraph were analyzed according to a derivation of the Scatchard equation to obtain dissociation constants and binding stoichiometry (*10*). Several anionic compounds were used as inhibitors of 5-CHO-H_4-PteGlu$_n$ binding. These anionic compounds have a low affinity compared to 5-CHO-H_4PteGlu$_n$. The values for K_i were determined by treating the Scatchard equation for the presence of a competitive inhibitor according to eqs 1 and 2

$$\frac{L_0}{\alpha} = K_{app}\frac{1}{1-\alpha} - E_0 \quad (1)$$

$$K_{app} = K_d + I_0\frac{K_d}{K_i} \quad (2)$$

where α is the fraction of saturation, I_0 is the total inhibitor concentration, L_0 is the total concentration of 5-CHO-H_4-PteGlu$_n$, and K_i is the competitive inhibition constant for the anion. The results of the titration were plotted as L_o/α versus $1/(1-\alpha)$. The slope of the line is K_{app} (eq 1). K_i was calculated using eq 2.

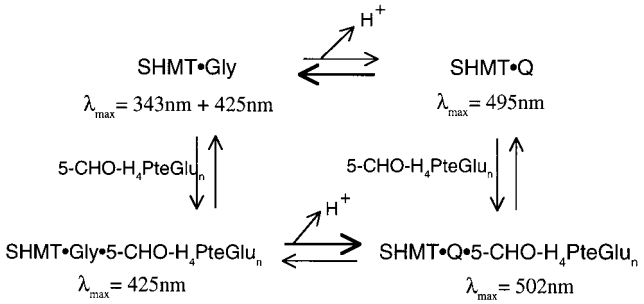
In the investigation of salt effects on 5-CHO-H_4PteGlu$_n$ binding, 20 mM potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate at pH 7.0, containing 50 mM glycine, was used in place of 20 mM potassium phosphate.

Isothermal Titration Calorimetry. Calorimetric measurements of the binding of 5-CHO-H_4PteGlu$_n$ were performed in an OMEGA titration microcalorimeter (Microcal, Inc., Northampton, MA). This instrument has been described in detail by Wiseman et al. (*11*). SHMT, about 6 mg/mL, was dialyzed overnight against 20 mM potassium phosphate buffer at pH 7.0, containing 50 mM glycine and 1 mM DTT. This enzyme was then diluted to the desired concentration with the dialysis buffer. A concentrated solution of 5-CHO-H_4PteGlu$_n$ was passed through a 1 cm × 10 cm Bio-Gel P-2 column equilibrated with dialysis buffer. The concentration of the solution was determined from its absorbance at 288 nm using an extinction coefficient of 31 500 M^{-1} cm^{-1} (*12*). All solutions were filtered and thoroughly degassed by stirring them under vacuum before use. The enzyme concentrations were between 0.01 and 0.1 mM, and 5-CHO-H_4PteGlu$_n$ concentrations were chosen so that the enzyme would be close to saturation before the final injection. In a typical titration experiment, SHMT in the sample cell was injected 12 times with microliter aliquots of 5-CHO-H_4-PteGlu or 5-CHO-H_4PteGlu$_2$ while the stirrer mounted on the injection syringe was operating at 300 rpm. Each injection lasted 1 min, and the injections were made 5 min apart to ensure the solution reached equilibrium before the next injection. A control experiment of the ligand titrated against buffer with no protein was also performed under identical conditions. The peaks of the obtained thermograms were integrated using the software supplied with the instrument, resulting in the heat released or absorbed from each injection. The data, after subtraction of the control signals, were analyzed with a nonlinear regression fitting program to obtain the dissociation constant of 5-CHO-H_4PteGlu$_n$ (K_d), the heat of binding (ΔH), and the binding stoichiometry (n). To calculate the binding heat capacity (ΔC_p) of 5-CHO-H_4-PteGlu$_n$, the titrations were performed at different temperatures from 20 to 40 °C.

The enthalpy changes determined by the binding of 5-CHO-H_4PteGlu$_n$ with an n of >2 were determined using a one-injection determination technique (*11*). A 10−20 μM SHMT solution was injected once with 20−30 μL of the ligand at a concentration that would ensure saturation of all binding sites. The heat released from the one injection was large enough to be accurately measured.

Location of the Polyglutamate Binding Site. The glutamate moiety of 5-CHO-H_4PteGlu was activated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (*13*). This complex was found to irreversibly inactivate rabbit liver cytosolic SHMT as well as *N. crassa* cytosolic SHMT and recombinant human liver cytosolic SHMT. The inactivation was performed with a SHMT:(6*S*)-5-CHO-H_4PteGlu:carbodiimide ratio of 1:10:100. Eighty-five microliters of a freshly made carbodiimide solution in 20 mM potassium phosphate buffer (pH 6.0) was mixed with 15 μL of 5.2 mM 5-CHO-H_4PteGlu in the same buffer. After a 5 min incubation at room temperature, a 25 μL aliquot of 8.4 mg/mL SHMT was added to this carbodiimide-activated 5-CHO-H_4PteGlu solution and 10 μL aliquots were removed at 10 min intervals to determine catalytic activity (*5*). Inactivation protection experiments were performed by adding 10 μL of a 0.33 mM SHMT solution to 10 μL of 0.45 mM 5-CHO-H_4PteGlu$_5$ prior to the addition of a 1−4-fold excess of an activated 5-CHO-H_4PteGlu solution in a 130 μL volume. Aliquots of 5 μL were removed to determine the remaining activity. A control experiment without preincubation with 5-CHO-H_4PteGlu$_5$ and a control experiment without addition of activated 5-CHO-H_4PteGlu were also performed.

Scheme 1



Identification of the amino acid residue irreversibly linked to 5-CHO-H_4PteGlu and 5-CHO-H_4PteGlu$_5$ was accomplished as follows. Mercaptoethanol was removed prior to the coupling reaction by passing the enzyme through a G-25 Sephadex column. Twenty microliters and 2.7×10^7 cpm of 5-[3′,5′,7,9-^{3}H]CHO-H_4PteGlu were mixed with 60 μL of 13 mM 5-CHO-H_4PteGlu and 10 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. After a 5 min incubation at room temperature, the activated 5-CHO-H_4PteGlu solution was added to 8 mg of SHMT in 0.7 mL of 20 mM potassium phosphate (pH 7.0). The coupling reaction was allowed to proceed for 30 min at room temperature. During this incubation, another aliquot of 3.9 μmol of unlabeled carbodiimide-activated 5-CHO-H_4PteGlu in 0.32 mL was prepared and added to the protein solution. This solution was allowed to incubate for another 30 min. The reaction was stopped by the separation of the free ligand from the protein by chromatography on a 0.7 cm × 15 cm G-25 Sephadex column equilibrated with 20 mM phosphate (pH 7.0). One milliliter fractions were collected and 20 μL aliquots counted. The fractions containing the protein were pooled. Cross-linking of 5-^{14}CHO-H_4PteGlu$_5$ was carried out in the same way as described for 5-CHO-H_4PteGlu. The labeled enzymes were denatured by dialysis against 8 M urea. The cysteine residues were blocked with iodoacetate as described previously (*15*). The proteins were then dialyzed against 0.1 M NH_4HCO_3 at pH 8.0 and digested with a 1% solution of trypsin for 8 h at 25 °C. The tryptic peptides were purified under the conditions described and the radioactive peptides analyzed by amino acid sequencing (*15*, *16*).

RESULTS

Spectrophotometric Determination of 5-CHO-H_4PteGlu$_n$ Binding to SHMT. Purified rabbit liver cytosolic SHMT is a tetrameric protein, and each subunit contains a pyridoxal phosphate molecule bound as an internal aldimine at the active site (*14*). In the presence of saturating amounts of glycine, three enzyme complexes can be identified by their spectral characteristics. These include a geminal diamine complex absorbing at 343 nm, an external aldimine absorbing at 425 nm, and a quinonoid complex absorbing at 495 nm (*14*). The predominate complex at pH 7.3 is the external aldimine with the geminal diamine representing about 25% of the total enzyme. The quinonoid complex is present in very small amounts with less than 1% of the enzyme in this form. The geminal diamine and external aldimine complexes are represented as the SHMT·Gly complex in Scheme 1, and the quinonoid complex is represented as SHMT·Q. In forming the quinonoid complex, the *pro-2S* proton of glycine is transferred to an unkown base on the enzyme which is in equilibration with solvent protons (*17*, *18*).

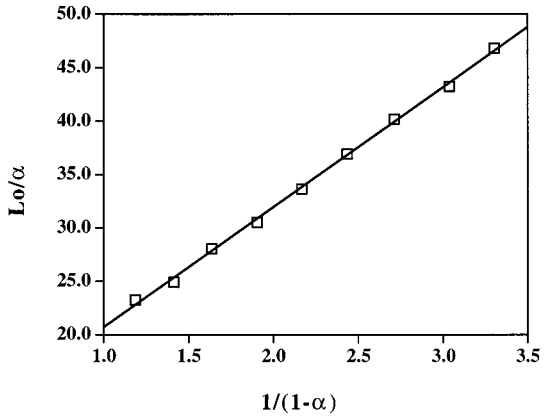


FIGURE 1: Titration of rabbit liver cytosolic SHMT with 5-CHO-H_4PteGlu. The binding of 5-CHO-H_4PteGlu was monitored at 502 nm as described in Experimental Procedures. The data were analyzed with a derivation of the Scatchard equation, $L_0/\alpha = K_d/(1 - \alpha) + E_0$, where L_0 is the total ligand added into the cuvette, α represents the fractional saturation of the enzyme, as determined by ΔA_{502}/maximalΔA_{502}, and E_0 represents the concentration of total ligand binding sites (*10*). The slope and *y*-intercept of the plot of L_0/α vs $1/(1 - \alpha)$ are K_d and E_0, respectively, and the latter divided by the enzyme concentration gives the value of stoichiometry *n*.

The addition of either H_4PteGlu$_n$, 5-CH_3-H_4PteGlu$_n$, or 5-CHO-H_4PteGlu$_n$ to the SHMT·glycine binary complex has been shown to shift the equilibrium toward the quinonoid complex SHMT·Q·H_4PteGlu (Scheme 1) (*4*). This quinonoid complex shows intensive absorbance at around 500 nm. Molar absorbtivity coefficients of 40 000 to 50 000 M^{-1} cm^{-1} have been reported for these complexes (*3*, *4*). This intense absorption of the quinonoid complex has been used to determine the dissociation constants of both glycine and the coenzyme (*2*, *3*, *19*). We took advantage of the stability of 5-CHO-H_4PteGlu$_n$, as opposed to the instability of both 5-CH_3-H_4PteGlu$_n$ and H_4PteGlu$_n$, to investigate the thermodynamic properties of coenzyme binding.

The dissociation constants and stoichiometry of binding of 5-CHO-H_4PteGlu$_n$ to rabbit liver cytosolic SHMT were determined by monitoring absorbance changes at 502 nm when the enzyme solution in 50 mM glycine at pH 7.0 was titrated with the mono-, di-, tri-, tetra-, and pentaglutamate forms of 5-CHO-H_4PteGlu$_n$. The K_d values for glycine are 6 mM for the binary complex and 0.8 mM for the ternary complex (*14*). Under the conditions used in this study, the enzyme was always completely saturated with glycine. Figure 1 shows a typical titration analyzed with a derivation of the Scatchard equation (*10*). The linear relationship suggests only one type of binding site exists and that each site is independent. The slope and the *y*-axis intercept represent the dissociation constant and the total ligand binding site concentration, respectively. Dividing the latter by the enzyme concentration allows the determination of binding stoichiometry, *n*. For each ligand, the titration was repeated three times and the values for K_d are shown in column 3 of Table 1. The majority of the decrease in K_d for 5-CHO-H_4PteGlu$_n$ occurred with the addition of the first two glutamate residues.

The stoichiometry of binding of 5-CHO-H_4PteGlu$_n$ to SHMT was also determined. For each glutamate chain length, the stoichiometry was near 0.5 per subunit. Since

Table 1: Thermodynamic Parameters for the Binding of 5-CHO-H_4PteGlu$_n$ to Rabbit Liver Cytosolic Serine Hydroxymethyltransferase at 25 °C

5-CHO-H_4PteGlu$_n$	K_a (μM^{-1})	K_d (μM)	ΔG (kcal/mol)[a]	ΔH (kcal/mol)[b]	$T\Delta S$ (kcal/mol)	ΔC_p (kcal mol^{-1} K^{-1})	n subunit
5-CHO-H_4PteGlu$_1$	0.17	5.9 ± 0.02	−7.1	8.8	15.9	−0.61	0.62 ± 0.07
5-CHO-H_4PteGlu$_1$	**0.15 ± 0.03**	6.7	−7.0	**7.6 ± 0.14**	14.6		**0.60 ± 0.02**
5-CHO-H_4PteGlu$_2$	1.72	0.58 ± 0.03	−8.5	9.0	17.5	−0.62	0.59 ± 0.09
5-CHO-H_4PteGlu$_2$	**2.2 ± 0.4**	0.45	−8.6	**7.7 ± 0.25**	16.3		**0.58 ± 0.05**
5-CHO-H_4PteGlu$_3$	14.7	0.068 ± 0.01	−9.7	5.9	15.6	−0.40	0.55 ± 0.07
5-CHO-H_4PteGlu$_4$	28.6	0.035 ± 0.003	−10.1	7.0	17.1	−0.42	0.51 ± 0.02
5-CHO-H_4PteGlu$_5$	50.0	0.020 ± 0.002	−10.5	5.4	15.9	−0.35	0.58 ± 0.03

[a] The values were calculated using $\Delta G = -RT \ln K_a$ and $K_a = 1/K_d$, where K_d values were obtained in the spectrophotometric study. Those in bold type were determined by ITC from a titration of SHMT with increasing concentrations of 5-CHO-H_4PteGlu$_n$. [b] The values in bold type for 5-CHO-H_4PteGlu$_1$ and -Glu$_2$ were determined from a titration of SHMT with increasing concentrations of 5-CHO-H_4PteGlu$_n$. The remaining values were determined from a single addition of a large excess of 5-CHO-H_4PteGlu$_n$ to SHMT as described in Experimental Procedures. The results are the average of duplicate trials which differed by less than 5%.

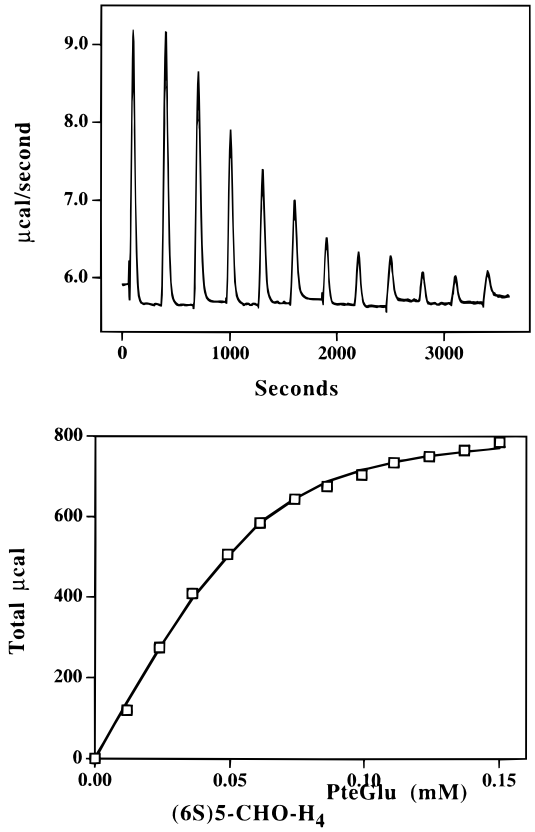


FIGURE 2: Isothermal titration calorimetry of rabbit liver cytosolic SHMT with (6*S*)-5-CHO-H_4PteGlu [thermogram (top) and isotherm (bottom)]. The solid line for the isotherm is a fit by the software of the instrument for an independent binding event with calculation of K_d and stoichiometry n.

the enzyme is a tetramer, it suggests that only two subunits bind 5-CHO-H_4PteGlu$_n$.

The dissociation constants and stoichiometry of 5-CHO-H_4PteGlu$_n$ binding were also determined at 15 and 35 °C. No significant differences from the K_d values and stoichiometry determined at 25 °C were observed (data not shown).

Isothermal Titration Calorimetry. The spectrophotometric study shows that a longer glutamate chain length increases the affinity of 5-CHO-H_4PteGlu$_n$ for SHMT by about 300-fold. Whether this increased affinity is attributed to changes in enthalpy or entropy was addressed by calorimetry. Isothermal titration calorimetry has been shown to be a good method for obtaining thermodynamic parameters of ligand binding to proteins (*11*, *20*).

We have determined entropy and enthalpy changes for the binding of 5-CHO-H_4PteGlu and 5-CHO-H_4PteGlu$_2$ to rabbit liver cytosolic SHMT using a stepwise titration technique and the enthalpy change for 5-CHO-H_4PteGlu$_n$ with n equal to 1−5 using the one-injection ΔH determination technique.

Figure 2 shows a typical stepwise titration thermogram (top) and the corresponding isotherm with a nonlinear regression fit (bottom) where SHMT was injected 12 times with microliter aliquots of 5-CHO-H_4PteGlu. The integrated area under each peak in the thermogram represents the heat effect upon each injection. The positive values show that the binding of 5-CHO-H_4PteGlu to SHMT is an endothermic process. Nonlinear regression fitting of the data of total heat versus total ligand concentration in the cell allows the determination of the association constant (K_a), enthalpy change (ΔH), binding stoichiometry (n), and entropy change (ΔS) derived from the equation $\Delta G = \Delta H - T\Delta S = RT \ln K_a$. Table 1 (rows 2 and 4, bold type) shows the automatic nonlinear regression fitting results for 5-CHO-H_4PteGlu and 5-CHO-H_4PteGlu$_2$ binding, where the values are the mean with the standard deviation of three titrations under the same conditions. The K_d values of 6.7 and 0.45 μM calculated from the K_a values for 5-CHO-H_4PteGlu and 5-CHO-H_4-PteGlu$_2$ binding using the relationship $K_d = 1/K_a$ are consistent with the K_d values of 5.9 and 0.58 μM determined by spectrometry, respectively (Table 1). The binding stoichiometry values of about 0.5 per subunit, determined by this method, are consistent with the values determined by spectrometry (Table 1, column 7). The positive ΔH values show that the binding process is enthalpically unfavored and that binding is driven by the favorable positive change in entropy.

The binding constant can be obtained optimally from a titration thermogram only if the product of the binding constant and protein concentration in the cell, K_a[protein], is in the range of 1−1000, preferably 10−100 (*11*). For 5-CHO-H_4PteGlu$_n$ with an n of >2, K_a is greater than 10^7. The protein concentration which allows K_a[protein] to fall into the preferred window is so low that the heat released from each injection could not be measured accurately. Thus, we could not determine the binding dissociation constant using the stepwise titration technique for 5-CHO-H_4-PteGlu$_{3-5}$. However, we could obtain ΔH by using a one-injection ΔH determination technique (see Experimental Procedures). These values are recorded in column 5 of Table 1. The values for ΔH determined for 5-CHO-H_4PteGlu$_{1-2}$

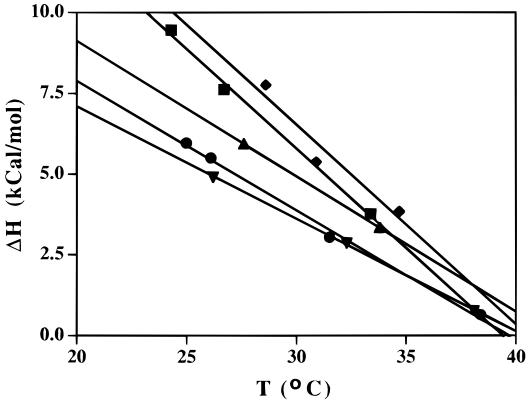


FIGURE 3: Variation of ΔH with temperature for formation of the cytosolic SHMT·Gly·5-CHO-H_4PteGlu$_n$ complex with n equal to 1 (■), 2 (◆), 3 (●), 4 (▲), and 5 (▼) as determined by calorimetry. The lines were drawn by linear regression analysis.

Table 2: Anion Inhibition of the Binding of 5-CHO-H_4PteGlu$_5$ to Rabbit Liver Cytosolic Serine Hydroxymethyltransferase

compound	concentration (mM)	K_i (mM)[a]
NaCl	25	15.4 ± 1.7
NaCl	100	11.7 ± 2.7
KCl	100	25
$N(CH_3)_4Cl$	100	27
malonate	25	24.9 ± 0.9
succinate	25	14.0 ± 2.0
glutarate	25	14.4 ± 3.5
pimelate	25	16.8 ± 1.8
suberate	25	15.1 ± 2.3
γ-Glu$_5$	1	0.42 ± 0.02

[a] The K_i values were calculated using eqs 1 and 2.

by this method are slightly higher than those determined by the stepwise titration experiments shown in bold type in Table 1. However, the difference in values does not affect the overall interpretation of what is controlling binding.

The enthalpy changes of the binding of 5-CHO-H_4PteGlu$_n$ were determined at four temperatures between 20 and 37 °C. A plot of ΔH versus temperature gave linear plots with negative slopes (Figure 3). The slopes of the linear fits allow the determination of ΔC_p, which vary from −0.61 to −0.35 for mono- to pentaglutamate forms, respectively (Table 1).

Ionic Strength Effects on Polyglutamate Binding. The effects of both cations and anions on 5-CHO-H_4PteGlu$_5$ binding were studied by determining the value of K_{app} (eq 1) in the presence of various ions at different concentrations using the spectrophotometric method. The buffer used in this study was 20 mM potassium *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate and 50 mM glycine (pH 7.0). Salts, such as NaCl, have K_i values of >200 mM when they were tested against 5-CHO-H_4PteGlu. However, salts are effective inhibitors of 5-CHO-H_4PteGlu$_5$, suggesting they are competing with the polyglutamate chain of the coenzyme for cationic sites on the enzyme. We have treated anion binding as a competitive inhibitor which should give a value for K_i that is independent of the concentration of the anion. This assumption of competitive inhibition is supported by only a small change in the value of K_i determined for 25 and 100 mM NaCl (Table 2). The use of other cations, such as potassium ion and tetramethylammonium ion, increased the value of K_i only slightly, suggesting that we are observing anion effects on inhibition (Table 1). To determine if there was an important spacing between the cationic groups on the enzyme, we determined the K_i values for a series of dicarboxylic acids. As shown in Table 1, the spacing of the anionic group did not appreciably affect the affinity for the anions for the polyglutamate binding site. We also tested the polyglutamate chain without the 5-CHO-H_4Pte group as an inhibitor. The K_i value for γ-linked pentaglutamate had a significantly higher affinity than other mono- or dianions (Table 2).

Table 3: Locus of Modified Lysine Residues of Rabbit Liver Cytosolic, Recombinant Human Liver Cytosolic, and *N. crassa* Cytosolic SHMTs Cross-Linked with (6*S*)-5-CHO-H_4PteGlu and (6*S*)-5-CHO-H_4PteGlu$_5$

Rabbit SHMT	**R** A T L **K** E ····F K_{450}* E **K** L A G D E **K H** Q **R** A V **R** A L **R**
Human SHMT	**R** A T L **K** E ····F K_{449}* E **R** L A G D E **K** Y Q **R** A V **R** A L **R**
N. crassa SHMT	**K** E A N **K** Q **K** D F K_{441}* A **K** I A T S D I P···**R** N E·····L **R**

SHMT Polyglutamate Binding Site. The polyglutamate binding sites on rabbit liver cytosolic SHMT, *N. crassa* cytosolic SHMT, and recombinant human liver cytosolic SHMT were located by covalent cross-linking the glutamate moiety of 5-CHO-H_4PteGlu and 5-CHO-H_4PteGlu$_5$ to the enzymes. The carboxyl groups were activated by water-soluble 1-ethyl-3-[(dimethylamino)propyl]carbodiimide prior to the addition of the proteins (*13*). The modification was found to irreversibly inactivate SHMT activity. The inactivation could be prevented when the enzyme was preincubated with 5-CHO-H_4PteGlu$_5$ prior to the addition of carbodiimide-activated 5-CHO-H_4PteGlu. With a ratio of 10:1 of activated 5-CHO-H_4PteGlu:SHMT and a 30 min reaction time, 90% of the SHMT activity was lost while a loss of activity of less than 10% could be accounted by the presence of only carbodiimide in the solution. These results suggest that the carbodiimide-activated 5-CHO-H_4PteGlu is binding at the folate site on SHMT.

Identification of the modified amino acid residues was accomplished by labeling the enzymes with either ^{3}H-labeled 5-CHO-H_4PteGlu or synthesized 5-^{14}CHO-H_4PteGlu$_5$ (see Experimental Procedures). The same conserved Lys residue was identified as the cross-linking site for both monoglutamate and pentaglutamate forms of the coenzyme. The modified residues are lysine 450, lysine 449, and lysine 441 for rabbit liver cytosolic SHMT, recombinant human liver cytosolic SHMT, and *N. crassa* cytosolic SHMT, respectively (Table 3). These lysine residues are located in a consensus sequence rich in positively charged arginine and lysine residues.

DISCUSSION

Most folate-requiring enzymes have a significantly higher affinity for the polyglutamate forms of the coenzyme than for the monoglutamate derivative (*21*). How the polyglutamate chain increases the affinity for the enzyme is presumed to be through its interaction with positive charges on the surface of the enzyme. Several possible roles for polyglutamylation of folates have been proposed, as reviewed by Schirch and Strong (*21*). One function of the polyglutamate chain is to block export of the coenzyme from the cell. Another possible function is that the polyglutamate chain plays a role in channeling the coenzyme between different folate sites so that the coenzyme is not released into free bulk solvent in the cell during metabolism.

Support for channeling of reduced folylpolyglutamates is best understood for the bifunctional thymidylate synthase−dihydrofolate reductase of protozoa (*22*−*26*). The key to channeling in this bifunctional enzyme is electrostatic attraction between the polyglutamate chain of dihydrofolate, which is generated at the thymidylate synthase site, and a positive electrostatic surface over the 40 Å distance to the dihydrofolate reductase site (*22*). From the crystal structure of the bifunctional enzyme, it was concluded that the polyglutamate chain made few specific contacts with residues on the protein but rather was held by an electrostatic field.

We have previously provided kinetic evidence that SHMT is involved in channeling with two other folate-requiring enzymes (*2*, *27*). The ability to study in detail the thermodynamic properties of the binding of 5-CHO-H_4PteGlu$_n$ to SHMT provides an opportunity to determine what properties of the polyglutamate chain enhance the affinity for the active site. The instability of reduced folates makes measuring enthalpy with the direct calorimetric technique all but impossible. When SHMT is titrated with either H_4PteGlu or 5-CH_3-H_4PteGlu in a titration calorimeter, there is a burst of heat released followed by a slow and further increase in heat released that takes several hours to come to equilibrium. Only 5-CHO-H_4PteGlu$_n$ gives reproducible measurements by isothermal titration calorimetry. We have taken advantage of this molecule binding to SHMT with about the same affinity as H_4PteGlu$_n$ to investigate the thermodynamic parameters involved in binding both the monoglutamate and the polyglutamate forms of this analogue. An additional feature of 5-CHO-H_4PteGlu$_n$ binding is that there is a simple and accurate spectrophotometric method of determining the value of K_d.

To determine thermodynamic values, the enthalpy for ligand binding must be either positive or negative. We tested several of our SHMT preparations from different sources and observed that both human cytosolic SHMT and rabbit mitochondrial SHMT gave very small positive values for ΔH. They were so small that it precluded doing any titrations to determine the values of K_a and stoichiometry by calorimetry. However, with rabbit cytosolic SHMT, the value for ΔH of binding 5-CHO-H_4PteGlu$_n$ was large enough that meaningful titrations could be performed, at least with the mono- and diglutamate derivatives. Because binding became so tight with longer glutamate chains, we could not do calorimetric titrations. This problem was circumvented by determining the values of K_d with these longer glutamate chains by a spectrophobmetric method and ΔH by calorimetry after a single addition of a saturating concentration of 5-CHO-H_4PteGlu$_n$.

The binding of 5-CHO-H_4PteGlu$_n$ to SHMT is a complex process involving release of a proton (Scheme 1) and a marked increase in the thermal stability of SHMT. Previous studies have shown that binding of 5-CHO-H_4PteGlu$_n$ to rabbit cytosolic SHMT increases the T_m from 67 to 81 °C (*28*). This suggests that some change in structure of the protein has taken place. However, it is clear from the data presented in Table 1 that the binding of 5-CHO-H_4PteGlu$_n$ is entropy-driven. Even for the human cytosolic SHMT and rabbit mitochondrial SHMT where ΔH is small and positive, this would also be true. The results can be interpreted as the binding of the pteridine ring and benzoyl group in hydrophobic pockets with the release of ordered water. A conformational change that folds in a hydrophobic patch on the surface releasing ordered water may also be involved. The involvement of hydrophobic interactions is supported by negative values of ΔC_p (Figure 3 and Table 1) (*29*−*31*). The large positive value for $T\Delta S$ most likely result from the release of ordered water from these hydrophobic surfaces. The negative values for ΔC_p also explain the observation that the value of K_d does not vary with temperature, showing that there is enthalpy−entropy compensation with changes in temperature.

The addition of glutamate residues to 5-CHO-H_4PteGlu$_n$ increases the affinity by 300-fold for SHMT. Most of this increase in affinity occurs with the addition of the second and third glutamate residues (100-fold decrease in K_d, Table 1). The values of ΔH for 5-CHO-H_4PteGlu$_1$ and 5-CHO-H_4PteGlu$_2$ were determined by both the titration method shown in Figure 2 and the single addition of an excess of each form of the coenzyme. The values for ΔH determined by the tirtration method are significantly higher than the values determined by the single addition method for both 5-CHO-H_4PteGlu$_1$ and 5-CHO-H_4PteGlu$_2$. This probably reflects the fact that we were not able to add saturating amounts of the mono- and diglutamate forms of the ligand in these experiments. The differences in ΔH values determined by the two methods should be closer for the tri- to pentagutamate forms where the K_d is very low and saturation can be obtained. However, this discrepancy between the values of ΔH by the two methods of determination provides a caution for the interpretation of why the polyglutamate forms of 5-CHO-H_4PteGlu$_n$ bind more tightly. With this precaution, the increase in affinity as glutamate residues are added appears to be the result of a negative ΔH of about 3 kcal/mol, resulting in a decreased positive ΔH for the entire structure (Table 1). This is most evident in the values in going from Glu_2 to Glu_3 (Table 1), where there is a 10-fold decrease in the value of K_d. However, the driving force for increased affinity as glutamate residues are added may be more complex than a simple change in enthalpy as indicated by the ΔH value for 5-CHO-H_4PteGlu$_4$ which is significantly higher than the ΔH values for either the Glu_3 or Glu_5 derivatives. There is little change in the values of entropy as glutamate residues are added. Even this constancy in entropy with increasing glutamate chain length could result from complex changes in binding such as off-setting effects of a positive ΔS for release of ordered water and a negative ΔS for loss of rotational freedom of the polyglutamate chain.

Unexpectedly, we observed a stoichiometry of 0.5 per subunit. We had previously shown that in the SHMT·Gly·5-CHO-H_4PteGlu$_n$ ternary complex 50% of the glycine was in the quinonoid complex (SHMT·Q) and 50% was in the external aldimine (*3*). We assumed that meant that the equilibrium constant between the aldimine and quininoid complexes was 1. However, it now appears that in the two subunits that bind 5-CHO-H_4PteGlu$_n$ glycine is completely in the quininoid complex and in the other two subunits glycine is bound as the external aldimine absorbing at 425 nm.

The increase in affinity upon addition of four additional glutamate residues results in a decrease in ΔG of 3.5 kcal/mol (Table 1). The ΔG for binding of the γ-linked pentaglutamate peptide is −4.5 kcal/mol (from the K_i value in Table 2). These two values are probably not significantly

different and suggest that the binding of the polyglutamate chain is an additive effect with the 5-CHO-H_4Pte part of the structure. The K_d and K_m values for binding of folylpolyglutamates to folate enzymes often vary when they are reported from different laboratories. This may reflect the sensitivity of binding to the buffer anion concentration. As shown in Table 2, a range of anions inhibit binding of 5-CHO-H_4PteGlu$_n$ to SHMT. This inhibition was not observed with the monoglutamate form of the coenzyme. The lack of a significant difference in the K_i values of a series of dicarboxylic acids with the negative charges separated by one to six methylene groups (malonate to suberate) suggests that the spacing of positive charges on the surface of the enzyme may not be critical. This evidence would suggest that the polyglutamate chain binds to a positive surface instead of having specific interactions between each glutamate carboxylate group with residues on the enzyme.

SHMT has been noted to contain a stretch of Arg and Lys residues in a region of predicted α-helix. Many other folate enzymes show similar stretches of positively charged amino acids.[2] A study on the inhibition of SHMT by Arg specific agents concluded that Arg-456 in this stretch was indeed involved in folate binding (Table 3) (*32*). We have cross-linked the carboxyl group of 5-CHO-H_4PteGlu with several of our SHMTs and found that Lys-450 in rabbit cytosolic SHMT and the corresponding residue in human cytosolic and *N. crassa* SHMTs are close enough to form an amide bond with the free carboxyl group of the coenzyme. This helps to further establish this stretch of Lys and Arg residues as being important in binding of the polyglutamate chain. We had hoped that additional binding sites in this stretch of positively charged residues could be identified by cross-linking of the pentaglutamate form of 5-CHO-H_4PteGlu$_n$. We added less than a stoichiometric amount of the carbodiimide reagent to 5-CHO-H_4PteGlu$_5$ for fear that if all the carboxyl groups were activated and there were no negative charged residues it may not bind specifically to the polyglutamate site. This left unaswered the question of which of the carboxylate groups of the pentaglutamate were activated by the carbodiimide. Another problem with this experiment was that the rate of reaction of a specific Lys residue on the protein will be different for each activated carboyxl group because of both differences in pK_a values of the different Lys residues and steric factors. Also, Arg residues in the sequence shown in Table 3 will not react with the activated carboyxl groups. Even with these problems, we had hoped to identify other Lys residues involved in the binding of the polyglutamate chain. However, the results showed that only Lys-450 was cross-linked to 5-CHO-H_4PteGlu$_5$ which is the same Lys that is cross-linked to the monoglutamate derivative. We will attempt to determine if the Lys and Arg residues near Lys-450 (Table 3) are a part of the polyglutamate binding site by changing these residue to non-charged amino acids by site-directed mutagenesis and determining the effect of the mutation on the affinity for 5-CHO-H_4PteGlu$_n$.

[2] Personal communication from R. Cook.

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