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# SERINE HYDROXYMETHYLASE

# SPECIFICITY OF BOND CLEAVAGE TO FORM QUINONOID INTERMEDIATES AND RATE OF HOLOENZYME FORMATION

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## Summary

L-Serine transhydroxymethylase (5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1) a pyridoxal phosphate-dependent enzyme, has been obtained as a homogeneous preparation with a specific activity of 6.7 μmol benzaldehyde per minute at 30°C at pH 7.5 in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, with DL-threo-β-phenylserine as a substrate.

This enzyme has been used to study the specificity of bond cleavage in forming quinonoid intermediates from DL and non-asymmetric amino acids. The ability of the generated quinonoids to react with formaldehyde and acetaldehyde has also been studied and evidence obtained for formation of the corresponding β-hydroxymethyl and β-hydroxyethyl amino acid derivaties.

Apotranshydroxymethylase has been prepared and the rate of holoenzyme formation was found to be 0.52 $min^{-1}$ by measuring Schiff base formation at 425 nm and 0.66 $min^{-1}$ as determined from restoration of enzymic activity. A requirement for the presence of mercaptoethanol for complete reactivation was also established by these studies.

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

## Introduction

Serine hydroxymethylase (5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1), a pyridoxal phosphate-dependent enzyme, catalyzes the L-serine-glycine interconversion shown in Reaction 1.

L-Serine + tetrahydrofolate ⇌ glycine + $N^5$,$N^{10}$-methylenetetrahydrofolate (1)

It was first reported to be present in sheep liver by Karasek and Greenberg [1] but the activity of their final preparation was not very high. Recently, Ulevitch and Kallen [2] reported a homogeneous preparation of the enzyme. The hydroxymethylase has been found to catalyze a variety of reactions: α,β-cleavage of β-hydroxy-L-amino acids, transamination with D-alanine, a tetrahydrofolate-dependent α-proton exchange of the Pro *s*-proton of glycine, and the decarboxylation of α-aminomalonate [3]. In the first three reactions the stereochemistry of bond cleavage is conserved. The hydroxymethylase from sheep liver has been reported to react with L-phenylalanine resulting in α-proton removal [2]. Using a high specific activity hydroxymethylase obtained in this laboratory by modifying the Ulevitch and Kallen procedures [2] we can now report that the enzyme is able to catalyze the removal of α-protons from L-alanine, L-tryptophan, and L-cyanoglycine. Action of the hydroxymethylase on these substrates represent a departure from the Cα bond cleavage specificity observed in other reactions catalyzed by this enzyme. Evidence is also presented that the carbanion generated by the hydroxymethylase and L-amino acids as substrates can react with formaldehyde and acetaldehyde to form the corresponding α-hydroxymethyl and α-hydroxyethyl amino acids.

Neither oxidized or reduced forms of folic acid affect the rate of formation nor stability of the quinonoid species generated from the hydroxymethylase and L-phenylalanine, L-alanine, L-tryptophan, or L-cyanoglycine.

Kinetics of holoenzyme formation and the reaction between the hydroxymethylase and α-cyanoglycine are also reported.

## Materials and Methods

Sheep livers were obtained fresh from Wilson Sinclair of Cedar Rapids, IA. Pyridoxal-5′-phosphate, folic acid, tetrahydrofolate, $N^5$-methylenetetrahydrofolate, DL-threo-β-phenylserine, L-threonine, DL-allothreonine, L-serine, alcohol dehydrogenase, NADH, $NADP^+$, $N^5$,$N^{10}$-methylenetetrahydrofolate dehydrogenase, L-phenylalanine, L-alanine, L-histidine, L-tryptophan, D- and L-chloroalanine, and glycine were purchased from Sigma. Ethyl acetamidocyanoacetic acid was purchased from Aldrich, L-tyrosine from Matheson, and Aquascint II and [$^{14}$C]formaldehyde from ICN.

Sheep liver serine hydroxymethylase was purified to a specific activity of 6.7 and $A_{280nm}/A_{415nm}$ ratio of 4.8 by a modification of the procedure described by Ulevitch and Kallen [2]. The procedures up to and including chromatography on phosphocellulose were the same. Beyond this point homogeneous enzyme by a number of criteria could be obtained by first subjecting the preparation to chromatography on Ultrogel AC-34 and finally on DEAE-Sephadex A-50.

The $\alpha$-$\beta$ cleavage of DL-threo-$\beta$-phenylserine to benzaldehyde and glycine was followed by monitoring the increase in the 279 nm absorption due to benzaldehyde production ($\epsilon_{279} = 1.41 \cdot 10^3\ M^{-1} \cdot cm^{-1}$). An enzyme unit is defined as the amount of enzyme that will produce 1 $\mu$mol benzaldehyde per min at 30°C at pH 7.5 in Hepes buffer. Specific activity is defined as the number of units per mg protein. Protein was determined by the method of Lowry et al. [4]. Spectra were recorded on a Beckman Model 24, AMINCO Model DW-2, and a Cary 118. Nuclear magnetic resonance spectra were obtained using a Varian H-60 NMR spectrometer. Radioactive counting was done on a Beckman L.S. 150 scintillation counter.

Tetrahydrofolate was prepared by $Na_2S_2O_4$ reduction of folic acid following the procedure of Davis [5]. Stock solutions of tetrahydrofolate and $N^5$-methyltetrahydrofolate were prepared fresh daily.

Stock solutions of L-phenylalanine, L-alanine, L-histidine, L-tyrosine, L-tryptophan, L-cyanoglycine and glycine were prepared in distilled water or potassium phosphate buffer. The pH of each solution was adjusted within the pH range 7.2—7.5 with 1 N KOH.

DL-Vinylglycine was synthesized according to the method of Rando [14], L-cyanoglycine according to the method of Ressler et al. [6], and DL-$\alpha$-methylphenylalanine according to the procedure of Stein et al. [7].

## Results

### *Formation of quinonoid species with L-amino acids by serine hydroxymethylase*

The reaction of serine transhydroxymethylase with L-alanine produces the visible spectrum shown in Fig. 1. It is characterized by the Schiff base absorption at 430 nm and a low intensity carbanion absorbing at 509 nm. L-Tryptophan reacts with enzyme to produce the spectrum also shown in Fig. 1, and is characterized by a Schiff base peak 430 nm and a carbanion of moderate intensity at 508 nm. Enzyme mixed with the L-isomers of histidine, tyrosine, methionine, $\beta$-chloroalanine, and 3,4-dihydroxyphenylalanine (dopa) shows no absorption in the carbanionic region of 480—510 nm.

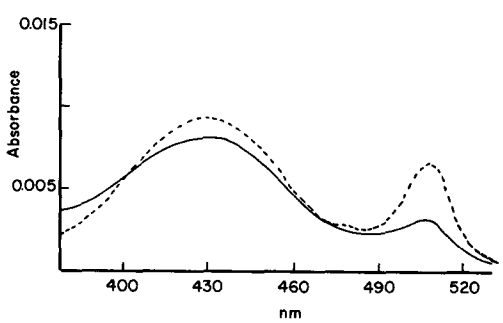


Fig. 1. Visible spectrum of serine hydroxymethylase and L-alanine (———) and in the presence of L-tryptophan (- - - - -). The samples contained 190 $\mu$g of enzyme in 1 ml 0.1 M L-alanaine or 0.05 M L-tryptophan at pH 7.5. The addition of $2 \cdot 10^{-5}$ mmol tetrahydrofolate produced no change in the spectrum.

All of the spectra recorded with serine hydroxymethylase and the L-isomers listed remained unchanged in the presence of $2 \cdot 10^{-5}$ mmol of either tetrahydrofolate or $N^5$-methyltetrahydrofolate.

L-Cyanoglycine, when bound by the hydroxymethylase, produces the visible spectrum shown in Fig. 2. The Schiff base (Fig. 2(1)) absorption exhibits a large loss in intensity while there is a very intense absorption at 481 nm (Fig. 2(2)) attributed to the enzyme-cyanoglycine carbanion and a small shoulder at approximately 455 nm.

*Formation of quinoid species by serine hydroxymethylase and D-amino acids*

D-Alanine reacts with serine hydroxymethylase as previously described [8] and its visible spectrum shows a Schiff base absorption and an absorption at 505 nm whose intensity is also dependent upon tetrahydrofolate. With D-alanine the hydroxymethylase catalyzes a transamination which results in the loss of the Schiff base absorption, and concomitant appearance of a peak at 325 nm due to pyridoxamine-5′-phosphate formation.

Other D-amino acids were tested to see whether they too could undergo $\alpha$-proton removal and transaminate in a manner analogous to D-alanine. Visible spectra of the D-amino acid-enzyme complexes were monitored (in the presence and absence of tetrahydrofolate) for changes in the Schiff base absorption, changes in the carbanion region of the spectrum (480—510 nm), and the appearance of any new absorptions. The possibility of $\alpha$-proton exchange in any of the enzyme-amino acid complexes was also monitored in the presence and absence of tetrahydrofolate of $N^5$-methyltetrahydrofolate by proton NMR using $^2H_2O$ as the solvent. Loss of enzymic activity, enzyme inhibition, and production of keto acids were monitored for the D-isomers of phenylalanine, phenylglycine, $\beta$-chloroalanine and the DL-pairs of vinylglycine and dopa. The tests showed negative results indicating that none of the D-amino acids tested mimicked the enzymic transamination observed with D-alanine.

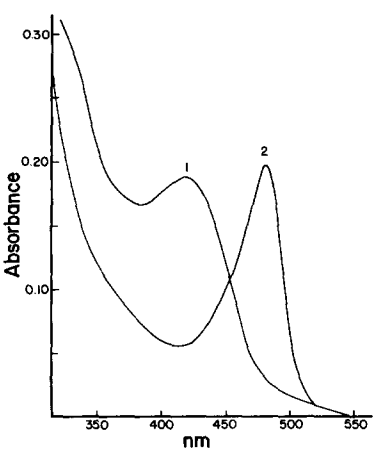


**Fig. 2. Visible spectrum of serine hydroxymethylase and L-cyanoglycine. The cuvette contained 420 μg of enzyme in 1 ml 0.08 M L-cyanoglycine (pH 7.5). Spectrum 1 is enzyme and spectrum 2 is enzyme plus cyanoglycine.**

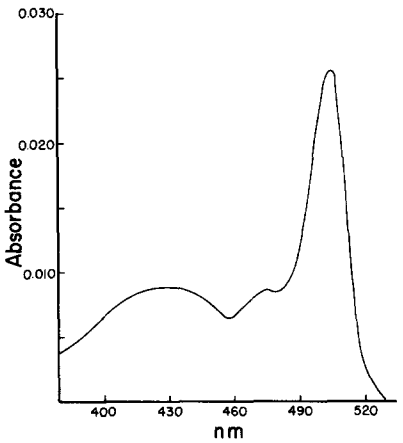


Fig. 3. Visible spectrum of serine hydroxymethylase and L-phenylalanine. The reaction mixture contained 190 μg of enzyme in 0.1 M L-phenylalanine at pH 7.5.

*Inactivation of serine hydroxymethylase by reaction of its quinonoids with aldehydes*

The 505 nm absorption generated by L-phenylalanine and serine hydroxymethylase (Fig. 3) can be quenched with formaldehyde or acetaldehyde. After the addition of 1.5 mg hydroxymethylase to 1 ml 0.1 M L-phenylalanine at pH 7.5, $6 \cdot 10^{-3}$ mmol formaldehyde were added. The absorption at 505 nm was quenched and an aliquot of this mixture showed no enzymic activity.

The cyanoglycine-hydroxymethylase carbanion absorbing at 481 nm (Fig. 2) and the glycine carbanions generated from α-aminomalonate and the hydroxymethylase are also quenched by formaldehyde.

*Formation of radioactive adducts between [$^{14}$C]formaldehyde and the quinonoid form of serine hydroxymethylase*

To assess if the carbanions generated from L-amino acids and serine hydroxymethylase would add to carbonyl groups, these species were reacted with [$^{14}$C]-formaldehyde. Serine hydroxymethylase (1.9 mg) was added to 1 ml 0.1 M L-phenylalanine and incubated for 30 min at 37°C, then 0.5 μmol of [$^{14}$C]-formaldehyde (10 mCi/mol) were added, the reaction sealed, and the mixture incubated at 37°C for an additional 30 min. The 1 ml enzyme sample was extensively dialyzed against distilled water and aliquots of the protein were then counted. No covalent attachment of the [$^{14}$C]formaldehyde to the enzyme was observed in spite of loss enzymic activity and quenching of the 505 nm absorption by the formaldehyde.

The production of an α-hydroxymethyl derivative of L-phenylalanine was detected in the following manner. To 0.1 ml 0.1 M L-phenylalanine (pH 7.3) was added 1.15 mg serine hydroxymethylase. The reaction mixture was incubated at 37°C for 30 min followed by the addition of 2 μmol [$^{14}$C]formaldehyde (1 mCi/mol). The sample was incubated for another 30 min at 37°C. The reaction was then stopped by the addition of 25 μl 1 N HCl, the precipitated protein was removed, and the supernatant applied to a Dowex 50

X-8 column. The unreacted [$^{14}$C]formaldehyde was eluted with 10 ml $H_2O$ and the $^{14}$C-labelled product was eluted with 5 ml 2 N $NH_4OH$.

The samples obtained contained 12 000, 15 000, and 21 000 cpm at 25, 30 and 40 min of incubation, respectively, when corrected for blanks containing no enzymes. These results demonstrated a linear increase in radioactive material, retained by Dowex 50 which could be eluted with 2 N $NH_4OH$, which was assumed to the radioactive amino acids.

Samples were prepared containing 23 μg hydroxymethylase, 2 μmol [$^{14}$C]-formaldehyde (1 mCi/mol), and 10 μmol aminomalonate in 0.2 ml 0.1 M phosphate buffer (pH 7.2). Samples containing 56 μg hydroxymethylase, 2 μmol [$^{14}$C]formaldehyde (1 mCi/mol) and 40 μmol L-cyanoglycine in 0.2 ml 0.1 M phosphate buffer (pH 7.2) were also prepared.

These samples were incubated 10 min at 37° C prior to [$^{14}$C]formaldehyde addition and then 20—30 min following [$^{14}$C]formaldehyde addition. Reactions were stopped by the addition of 25 μl 1 N HCl and the samples were applied to a Dowex 50 X-8 column. The unreacted [$^{14}$C]formaldehyde was eluted with 10 ml of distilled water, and the corresponding $^{14}$C-labelled products were eluted with 5 ml 2 N $NH_4OH$. The controls for aminomalonate and cyanoglycine were prepared in the same manner, but they contained no serine hydroxymethylase, and were subjected to the same procedure. Radioactivity corresponding to newly synthesized amino acids could be demonstrated by this procedure for hydroxymethylase and cyanoglycine or aminomalonate.

*Determination of the rate of holoenzyme formation for serine hydroxymethylase*

To determine the rate of holoenzyme formation apohydroxymethylase was prepared by a modification of the procedure used by Schirch et al. [9]. Serine hydroxymethylase was mixed with 1.0 M L-cysteine and then dialyzed against 0.1 M phosphate buffer (pH 7.3) containing 2 mM mercaptoethanol. The degree of reconstitution of holoenzyme was determined by monitoring the gain in enzymic activity from the incubation of apohydroxymethylase and pyridoxal-5′-phosphate. To 0.1 ml apoenzyme (1.76 mg/ml) incubated at 30° C was added 10 μl of pyridoxal-5′-phosphate (0.25—2.54 mM) at time zero. Aliquots (10 μl) were removed and assayed using DL-threo-β-phenylserine as substrate. Pseudo-first-order kinetics were observed, and the rate was dependent upon the pyridoxal-5′-phosphate concentration and the presence of thiol. The following scheme was used to determine the rate constants.

$$\text{Apoenzyme} + \text{pyridoxal phosphate} \overset{k}{\rightleftharpoons} \text{holoenzyme}$$

$$-\mathrm{d}\,\frac{\{\text{Apoenzyme}\}}{\mathrm{d}t} = k'\,\{\text{Apoenzyme}\};$$

$$k' = k\,\{\text{pyridoxal phosphate}\}$$

$$\ln\frac{Apo_x}{Apo_0} = k't$$

Experimentally the ratio of the enzymic activity at any time, $t$, to the original enzymic activity was the parameter determined. This is actually the ratio of holoenzyme to total enzyme ($E_t$), but assuming $Apo_x$ plus $Holo_x$ (the concentrations of apo- and holohydroxymethylase at any time, $t$) equals $E_t$, then $Holo_x/E_t = (E_t - Apo_0)/E_t$. $Apo_0$ (the concentration of apohydroxymethylase at time zero) = $E_t$ and, therefore, $(1 - Holo_x)/E_t = Apo_x/E_t(Apo_x)/(Apo_0)$.

The reconstitution of enzymic activity as a function of time and pyridoxal-5′-phosphate is shown in Fig. 4. The plot for determination of $k'$ is shown in Fig. 5. The value of $k'$ varies from 0.22 $min^{-1}$ at 0.46 mM pyridoxal-5′-phosphate, 0.28 $min^{-1}$ at 0.077 mM, 0.43 $min^{-1}$ at 0.231 mM to a value of 0.66 $min^{-1}$ at 0.468 mM.

The increase in absorption at 425 nm due to formation of the Schiff base was determined using the Cary 118 in the following manner.

Apohydroxymethylase (1 mg) was added to 50 $\mu$l 2.54 mM pyridoxal-5′-phosphate in a total volume of 0.55 ml 0.1 M phosphate buffer (pH 7.3) containing 2 mM mercaptoethanol. The increase in absorbance at 425 nm was monitored as a function of time against a blank containing the same concentration of pyridoxal-5′-phosphate. The ln $\Delta A_{425}$ was plotted against time, and the pseudo-first-order rate constant calculated was approx. 0.52 $min^{-1}$ as compared to a value of 0.66 $min^{-1}$ determined from enzymic activity measurements.

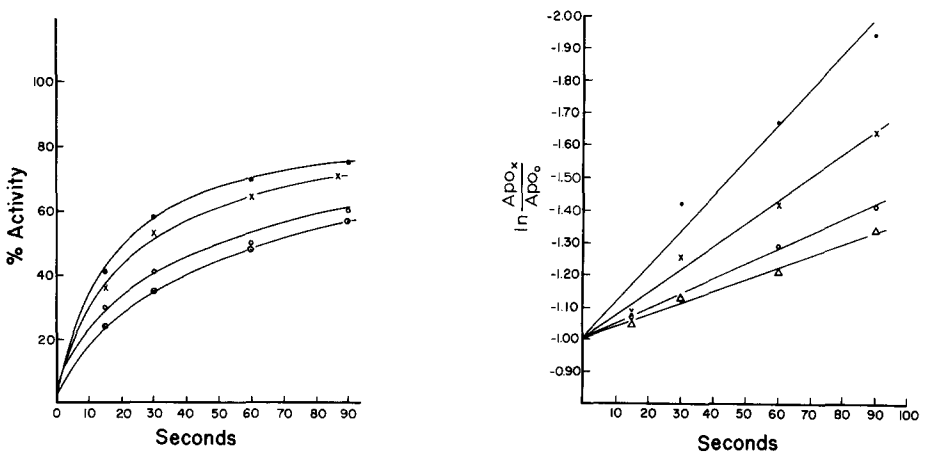


Fig. 4. Reconstitution of holoenzyme from apotranshydroxymethylase and pyridoxal-5′-phosphate. The concentrations of pyridoxal-5-′-phosphate are as follows: (●) 0.468 mM, (X) 0.308 mM, (○) 0.231 mM, and (⊙) 0.115 mM. The % activity is equal to the ratio of the enzymic activity at any time, $t$, to the enzymic activity of the unmodified holotranshydroxymethylase X100.

Fig. 5. Plot of the reconstitution of holotranshydroxymethylase as a function of time and pyridoxal-5′-phosphate concentration. Pseudo-first-order kinetics are observed. $Apo_x$ designates the concentration of aproenzyme at any time, $t$, and $Apo_0$ designates the initial concentration of apoenzyme at time zero. The pyridoxal-5′-phosphate concentrations are (●) 0.468 mM, (X) 0.231 mM, (○) 0.077 mM, and (△) 0.046 mM. All lines have been corrected to a value of —1.00 at time zero.

## Discussion

According to Jordon and Akhtar [10] and Besmer and Arigvoi [11] the stereochemistry of proton removal by serine hydroxymethylase is pro-S specific. The fact that serine hydroxymethylase forms a quinonoid species with D-alanine [8] is consistent in that the $\alpha$-proton of D-alanine is equivalent to the pro-S proton of glycine. Ulevitch and Kallen [2] first reported the unexpected observation that L-phenylalanine reacts with serine hydroxymethylase to form a quinonoid intermediate. In this manuscript we have used a high specific activity preparation of hydroxymethylase to extend their observation to other L-amino acids. It is now shown that L-alanine, L-tryptophan and L-cyanoglycine will also form quinonoid species with the sheep serine hydroxymethylase. These observations contradict Dunathan's theory that for a single $B_6$ requiring enzyme there should be conservation of C-$\alpha$ bond cleavage [12]. According to this theory the C-$\alpha$ bond exhibiting the greater probability of undergoing cleavage should be the one which would be perpendicular to the plane containing the imine, nitrogen and the pyridine ring. Maximum overlap of the $\pi$ system and the incipient $\sigma$ bond would occur, if that $\sigma$ bond occupied the perpendicular position. This interaction is illustrated in Fig. 6. The dotted line represents the $\pi$ system (the pyridine ring and imine nitrogen which are

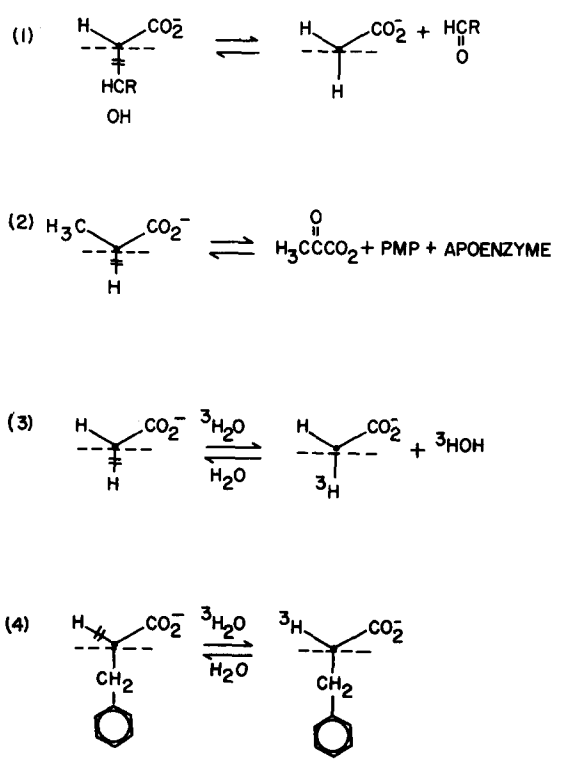


**Fig. 6. Stereochemistry of bond cleavage for serine transhydroxymethylase. The plane containing the pyridine ring and the imine nitrogen, which lies perpendicular to the paper, is represented by the dotted line. The hash marks denote the bond that is broken.**

coplanar) which is perpendicular to the plane of the paper. The bond that is cleaved is the one with the hash marks. In Reactions 1, 2, and 3 — the dealdolization of a β-hydroxy amino acid, the transamination of D-alanine, and the tetrahydrofolate-dependent exchange of the pro-S proton of glycine, respectively — with the amino and carboxyl groups occupying fixed positions, the bond that is cleaved can be positioned perpendicular to the plane as described. Clearly, Reaction 4, the exchange of the α-proton of L-amino acids, cannot 'fit' the stereochemical requirements of Dunathan.

The reaction of L-amino acids with serine hydroxymethylase to form carbanion clearly represents a break in the stereochemistry of bond cleavage. Carbanions formed by mixing each of the L-isomers of tryptophan, alanine, and cyanoglycine with the transhydroxymethylase represent anomalous stereochemistry.

We have also shown that the carbanions of L-phenylalanine, L-cyanoglycine, and aminomalonate react with $^{14}CH_2O$ in a manner analogous to the formation of serine from glycine and formaldehyde.

Previously, Meister et al. [13] have shown that the cytoplasmic hydroxymethylase isolated from rat liver catalyzes the non-stereospecific decarboxylation of α-aminomalonate. The sheep liver hydroxymethylase reacted with aminomalonate in a similar manner. Thus the aminomalonate reaction also exhibits an apparent anomalous stereochemistry. This reaction is especially interesting because both glycine carbanions are produced for the first time. The quenching of these carbanions with formaldehyde should produce both isomers of serine. This is currently under investigation in our laboratory.

In reference to the Dunathun postulate , all results with the L-amino acids could reflect the 'unfavourable' conformation that the α-hydrogens of L-phenylalanine and the other L-amino acids must assume when bound by the enzyme if they are to undergo proton abstraction.

Modified amino acids such as vinylglycine [14], chloroalanine [15], and cyanoglycine [16] have been observed to be both substrate and $k$/cat inhibitors of a number of pyridoxal-5′-phosphate requiring enzymes. With the serine hydroxymethylase only cyanoglycine gave a positive reaction. It is not a $k$/cat inhibitor for the serine hydroxymethylase, however, it does form a quinonoid intermediate with the enzyme. The carbanion absorption at 481 nm observed when cyanoglycine is mixed with serine hydroxymethylase presumably results from the removal of the α-proton of cyanoglycine. The quinonoid intermediate with cyanoglycine can be displaced by substrate. Therefore, one observes competitive inhibition of serine hydroxymethylase by cyanoglycine.

The reconstitution of serine hydroxymethylase follows pseudo-first-order kinetics which depends upon both the concentration of pyridoxal-5′-phosphate and the presence of mercaptoethanol. Holoenzyme regeneration to approx. 90% in 5 min was possible from apoenzyme dialyzed against phosphate buffer containing 2 mM mercaptoethanol. Reconstitution percentages obtained in the absence of thiol were only 5—9% of the percentages achieved in the presence of thiol. The addition of mercaptoethanol to apohydroxymethylase dialyzed against non-mercaptoethanol buffer followed by addition of pyridoxal-5′-phosphate resulted in a time-dependent reconstitution which appeared biphasic.

Two assumptions have been made concerning the determination of the pseudo-first-order rate constants: (1) the binding of pyridoxal-5′-phosphate to the apoenzyme results in the immediate formation of active hydroxymethylase; and (2) the Schiff base appearance at 425 nm, when apoenzyme and pyridoxal-5′-phosphate are mixed, is due to the formation of active enzyme. The possibility of structural changes occurring between the initial binding of pyridoxal-5′-phosphate and the initial appearance of active enzyme cannot be eliminated. In addition, the observations on reconstitution in the absence of thiol suggest that thiol is required to maintain the structural integrity of the apoenzyme towards reconstitution; but it cannot be shown by the techniques used in this study whether this decreased activity actually represents holoenzyme of low activity or a small percentage of holoenzyme still remaining in its native conformation.

## Acknowledgement

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