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Serine Transhydroxymethylase

SUBUNIT STRUCTURE AND THE INVOLVEMENT OF SULFHYDRYL GROUPS IN THE ACTIVITY OF THE ENZYME*

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SUMMARY

The results of peptide map patterns, end group analysis, and disc electrophoresis experiments in gels containing 8 M urea show that serine transhydroxymethylase from rabbit liver consists of four identical polypeptide chains.

Evidence is presented to show that serine transhydroxymethylase can exist in an oxidized form and a reduced form. The oxidized form is about 60% as active as the reduced form but can be converted to the reduced form by incubation with a sulfhydryl compound, such as dithiothreitol, and pyridoxal 5′-phosphate. In the absence of a sulfhydryl compound the reduced enzyme is converted to the oxidized enzyme over a period of several weeks at −5°. The apoenzyme is converted to the oxidized form in a few hours.

The reduced enzyme has 12 sulfhydryl groups which react with 5,5′-dithiobis(2-nitrobenzoic acid). Kinetically they are of two classes. Eight of these sulfhydryl groups react rapidly and four more slowly. The oxidized enzyme has only eight sulfhydryl groups which react with 5,5′-dithiobis(2-nitrobenzoic acid), four reacting rapidly and four more slowly. The reaction of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) causes dissociation of pyridoxal 5′-phosphate and complete loss of enzymatic activity.

We have previously shown that serine transhydroxymethylase from rabbit liver catalyzes the cleavage of several β-hydroxyamino acids to yield glycine and an aldehyde (1, 2). A considerable amount of work has been done on the mechanism of the reaction but little is known about the physical and chemical properties of the enzyme. Martinez-Carrion *et al.* have established that the enzyme is composed of four subunits and has a molecular weight of 215,000 (3). Fujioka has presented evidence that cytoplasmic and mitochondrial isozymes exist in rabbit liver and that the two enzymes are very similar in their physical and chemical properties (4). Several investigators, including ourselves, have been using serine transhydroxymethylase preparations purified by a procedure which has not been established to separate the two isozymes (2, 3, 5). Also, our preparations have consistently shown activation by pyridoxal-P[1] while neither of the isozymes purified by Fujioka were activated by this coenzyme. Our activation was dependent on the presence of a sulfhydryl compound, a fact that implicates the involvement of sulfhydryl groups at the active site of serine transhydroxymethylase.

With our interest in understanding the mechanism of serine transhydroxymethylase, we felt that it was necessary to establish if our preparations were a mixture of the two isozymes and to explain the sulfhydryl-dependent pyridoxal-P activation. Since Fujioka had already shown that the two isozymes have similar molecular weights and electrophoretic mobilities, we centered our attention on the properties of the subunits. NH_2- and COOH-terminal studies, peptide map analysis, and electrophoretic patterns in acrylamide gels containing 8 M urea suggest that our preparations are homogeneous and that the enzyme consists of four identical subunits.

We have also resolved the problem of why our purified preparations were activated by pyridoxal-P while the preparations of Fujioka show no such activation. Our studies show that serine transhydroxymethylase can exist in an oxidized form which has fewer titratable sulfhydryl groups and a lower activity than a reduced form of the enzyme. The oxidized enzyme is obtained if the purification is performed in the absence of a sulfhydryl-containing compound. The oxidized enzyme can be readily converted to the fully active reduced form by the addition of pyridoxal-P and a sulfhydryl compound. Fujioka routinely added β-mercaptoethanol and pyridoxal-P at each step in the purification which resulted in a preparation containing only the fully active reduced enzyme (4).

EXPERIMENTAL PROCEDURE

Materials—Serine transhydroxymethylase was purified from fresh frozen rabbit liver as previously described (6). Studies

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[1] The abbreviations used are: pyridoxal-P, pyridoxal 5-phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

were performed with enzyme which had been crystallized at least twice and which showed an $A_{280}:A_{430}$ ratio of 6.3. The enzyme gave one band on starch gel electrophoresis but sometimes a 1 or 2% impurity was evident in ultracentrifuge sedimentation patterns.

The activity of the enzyme was routinely assayed with allothreonine as the substrate (2). The product acetaldehyde was reduced with DPNH in the presence of alcohol dehydrogenase and the rate of the reaction determined by the decrease in absorbance at 340 nm.

All reagents used were of analytical grade and were purchased either from Sigma Chemical Co. or Carlo Erba.

Amino Acid Analysis—Serine transhydroxymethylase was hydrolyzed for 24, 37, 49, and 72 hours in 6 N HCl, containing one small crystal of phenol, at 110°. Analyses of the hydrolyzed materials were performed on a Bio-Cal BC 200 instrument, with a single column system. The amino acid compositions were determined and extrapolated when necessary to either zero or infinite hydrolysis time.

Total cystine and cysteine residues were evaluated as cysteic acid after 6 N HCl hydrolysis in the presence of 0.21 M dimethylsulfoxide (7).

The tryptophan content of the enzyme was determined spectrophotometrically by the method of Bencze and Schmid (8).

Amides were measured by the carbodiimide method of Hoare and Koshland (9). The reaction was performed in 6 M guanidine hydrochloride with 1-ethyl-3-dimethylaminopropyl carbodiimide as activating agent and β-alanine ethyl ester as nucleophile. Four samples were reacted for 31 to 55 hours and were found to give identical results.

NH_2- and COOH-terminal Amino Acids—The dansyl chloride method of Gray (10), the Edman reaction (11), and the cyanate method of Stark and Smyth (12) were used to determine the NH_2-terminal amino acid.

The quantitative determination of the COOH-terminal amino acid was made by treating reduced enzyme with ethyleneimine (13) and maleic anhydride (14). The soluble aminoethylated enzyme was incubated with diisopropyl fluorophosphate-treated carboxypeptidases A and B. At several time intervals aliquots were removed and analyzed with an amino acid analyzer. Norleucine was added to the reaction mixture to serve as an internal standard.

Peptide Map Analysis—Ten milligrams of enzyme in 0.5 ml of 0.1 M Tris chloride and 8 M urea, pH 8.0, were reduced with 1.0 ml of β-mercaptoethanol for 24 hours under nitrogen. The —SH groups were then reacted with an excess of acrylonitrile (4 times the β-mercaptoethanol concentration) for 4 hours at room temperature. An additional 4-fold excess of acrylonitrile was then added and allowed to react for an additional 4 hours. The solution was dialyzed overnight against 0.1 M ammonium bicarbonate. The enzyme with the blocked —SH groups formed a fine precipitate. Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated), 0.2 mg, was added to the enzyme solution and the mixture was incubated at 25° for about 3 hours with stirring. The reaction appeared to be complete but an additional 0.2 mg of trypsin was added and the incubation was continued for another 3 hours. A clear solution remained after digestion. The digested enzyme solution was lyophilized and the resulting powder dissolved in 0.2 ml of 0.1 M ammonium carbonate. Ten to twelve microliters were spotted on chromatographic paper and chromatographed in pyridine-1-butanol-acetic acid-water, 60:90:18:72. The chromatogram was dried and then subjected to electrophoresis in either 1% ammonium carbonate, pH 8.0, or pyridine-acetic acid-water, 1:10:89, at pH 3.5. After the paper was dry, it was sprayed with 0.3% ninhydrin in acetone.

Disc Acrylamide Gel Electrophoresis—Electrophoresis of serine transhydroxymethylase in 8 M urea at pH 8.4 and 3.1 was performed according to the method of Summaria *et al.* (15). The enzyme was incubated in 8 M urea and 10 mM β-mercaptoethanol for 24 hours prior to electrophoresis. Electrophoresis was carried out at 2.5 ma per tube for 4 hours.

Preparation of Apo-serine Transhydroxymethylase—To several milliliters of a 5 mg per ml enzyme solution were added $(NH_4)_2SO_4$ and L-cysteine to 30% and 0.1 M, respectively. This solution was then dialyzed for 8 hours against two changes of 5 volumes of 0.05 M potassium phosphate, pH 7.2, containing 30% $(NH_4)_2SO_4$ and 0.1 M L-cysteine. The dialyzed enzyme was then passed through a Sephadex G-75 column equilibrated with 0.01 M potassium phosphate, pH 7.2.

Moles of Pyridoxal-P per Mole of Enzyme—The pyridoxal-P content of serine transhydroxymethylase was determined by the cysteine method of Schirch and Mason (1) and the phenylhydrazine method of Wada and Snell (16).

Sulfhydryl Group Determination—The number of reactive —SH groups were determined by reaction with DTNB (17).

Protein Determination—Serine transhydroxymethylase exhibits absorption maxima at 428 and 277 nm (1). In this paper we determined the absorbance of a 1% solution at 277 nm ($E^{1\%}_{277\ nm}$) by two dry weight procedures. In the first procedure 30 mg of the pure enzyme were dialyzed against water for 2 days. The solution was lyophilized and dried under vacuum over P_2O_5 for 48 hours. A sample of the dried protein, 8.50 mg, was dissolved in 10 ml of 10 mM potassium phosphate buffer, pH 7.3, and the spectrum recorded. From the absorbance at 277 nm an $E^{1\%}_{277\ nm}$ of 7.2 was calculated from duplicate samples.

In the second procedure a 10 mg per ml solution of enzyme was dialyzed for 2 days against distilled water. An aliquot of the solution was diluted 10-fold in the phosphate buffer and the absorbance at 277 nm determined. Another aliquot, 2.0 ml, of the dialyzed solution was added to a preweighed vial and evaporated to dryness and constant weight at 105°. The $E^{1\%}_{277\ nm}$ obtained by this procedure was also 7.2.

RESULTS

Subunit Structure

Amino Acid Analysis—Table I summarizes the amino acid composition at four periods of hydrolysis. The values obtained are based on a molecular weight of 215,000 (3). The data show that more than half of the acidic amino acids are present as the amides. Also the basic amino acids arginine and lysine outnumber acidic acids aspartic acid and glutamic acid. This is in agreement with the observation that the enzyme behaves as a cation on ion exchange columns at pH values up to 7.3. The enzyme also migrates at pH 8.4 in electrophoresis systems as though it was only slightly anionic.

The calculation of the partial specific volume from the amino acid composition gave a value of 0.735 ml per g.

Peptide Map Analysis—The results of chromatography and electrophoresis of tryptic hydrolysates of serine transhydroxymethylase are shown in Fig. 1. About 28 peptides were consistently identifiable in both pH 3.5 and 8.0 electrophoresis systems. Since amino acid analysis indicates 150 lysine and arginine residues, the peptide map analysis suggests that the en-

TABLE I

Amino acid composition of serine transhydroxymethylase

Amino acid	Duration of hydrolysis				Nearest integer
	24 hrs	37 hrs	49 hrs	72 hrs	
Lys	71.8	79.3	74.2	71.8	74
Arg	75.7	75.7	79.6	75.7	76
His	41.7		41.7	41.7	42
Asp	112.0	113.5	117.4	113.5	114
Glu	153.5	151.4	153.3	151.4	152
Thr	56.7	55.3	52.9	47.9	62
Ser	68.2	56.8	52.9	45.4	83
Pro	94.6	98.5	90.7	83.2	92
Gly	124.9	124.9	124.9	124.9	125
Ala	124.9	121.0	124.9	109.7	120
½Cys					30
Val	79.6	79.6	83.2	83.2	83
Met	20.4	21.9	21.9	21.5	21
Ile	58.7	58.9	60.3	63.2	63
Leu	136.3	132.4	136.3	136.3	135
Tyr	56.8	49.2	49.2	49.2	49
Phe	45.4	45.4	45.4	45.4	45
Try					15[a]
Amides					159[b]

[a] Determined spectrophotometrically (8).

[b] Determined by the carbodiimide method (9).

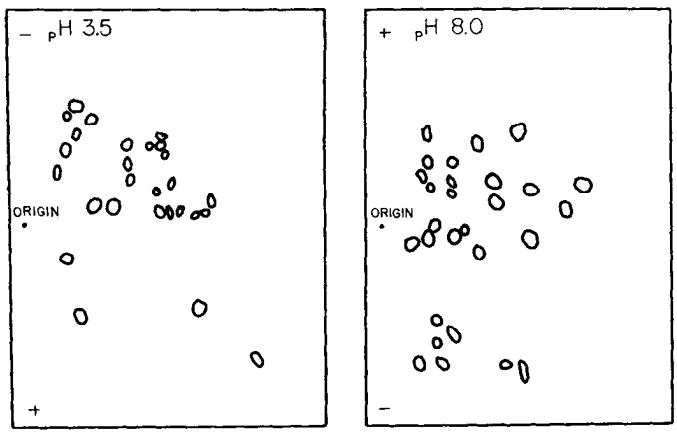

FIG. 1. Peptide map of tryptic digest of serine transhydroxymethylase. Ten milligrams of enzyme in 0.8 M Tris chloride and 8 M urea, pH 8.0, were reduced with 1.0 ml of β-mercaptoethanol for 24 hours under nitrogen. A 4-fold excess of acrylonitrile was added twice at 4-hour intervals. After dialysis overnight the enzyme was digested with 0.2 mg of trypsin. At the end of 3 hours an additional 0.2 mg of trypsin was added and the solution incubated for an additional 3 hours. The solution was chromatographed in pyridine-1-butanol-acetic acid-water, 60:90:18:72, and the chromatogram was then subjected to electrophoresis in either 1% ammonium carbonate, pH 8.0, or pyridine-acetic acid-water, 1:10:89, at pH 3.5. The dried chromatograms were developed with 0.3% ninhydrin in acetone.

zyme consists of at the most six identical polypeptide chains. Molecular weight studies have shown that the enzyme consists of four subunits which indicates that the peptide map analysis did not resolve all of the peptides. This study, however, supports the view that the four subunits are identical.

End Group Analysis—The dansyl chloride method revealed the presence of glycine as the NH_2-terminal amino acid. The Edman reaction revealed the presence of glycine and a small amount of proline. In order to determine the relative amounts

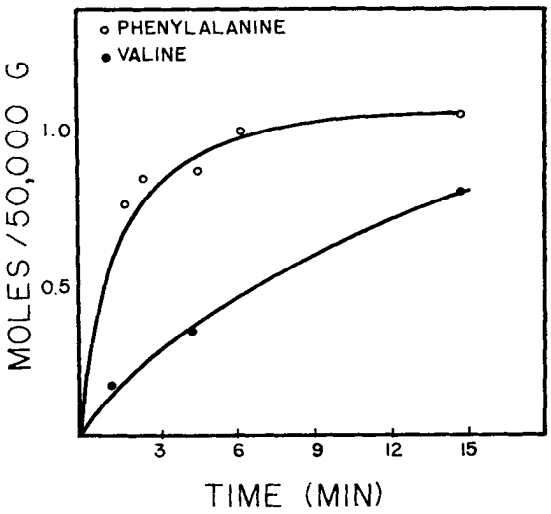

FIG. 2. Amino acids released during digestion of serine transhydroxymethylase with carboxypeptidases A and B. The enzyme was reduced and aminoethylated (13) and then treated with maleic anhydride (14). During incubation with carboxypeptidases A and B aliquots were removed and analyzed for released amino acids, as described in the text.

of glycine and proline, a quantitative determination was attempted by the use of the cyanate method. This method did not give reproducible results. Although all experiments showed that glycine is the major NH_2-terminal amino acid, the amount of proline ranged from none to as high as 22% of the amount of glycine. In our best experiment the glycine content was 1 mole of glycine per 50,000 g of protein.

Digestion of serine transhydroxymethylase in sodium dodecyl sulfate with either carboxypeptidase A or B released only phenylalanine as the COOH-terminal amino acid. The quantitative determination of the COOH-terminal amino acid was obtained by hydrolysis of the aminoethylated enzyme by carboxypeptidases A and B. As shown in Fig. 2, 1 mole of phenylalanine was released for each 50,000 g of protein. The data in Fig. 2 suggest that valine is the penultimate amino acid.

Acrylamide Gel Electrophoresis—If all four subunits are identical, a single band should be obtained in electrophoresis systems in which the enzyme is dissociated into its monomers. It has previously been shown that in 1% sodium dodecyl sulfate and 10 mM β-mercaptoethanol serine transhydroxymethylase migrates as a single band with a molecular weight of 53,000 (3). We have verified this finding with our preparations.

Serine transhydroxymethylase, incubated in 8 M urea and 10 mM β-mercaptoethanol, was subjected to acrylamide disc gel electrophoresis at pH 8.4 and 3.1. In each case the gels were made in the presence of 8 M urea. The electrophoresis patterns show a single band at each pH.

Sulfhydryl Group Involvement in Activity of Serine Transhydroxymethylase

Evidence for Oxidized and Reduced Forms of Enzyme—We feel that the following observations support the interpretation that serine transhydroxymethylase can exist in two forms which we shall call the oxidized enzyme and the reduced enzyme. The specific activity of the oxidized enzyme is about 60% of the activity of the reduced enzyme.

Enzyme purified either in the presence or absence of pyridoxal-P, but in the absence of a thiol compound, always gives an enzyme preparation which can be activated by the

addition of pyridoxal-P and dithiothreitol, or β-mercaptoethanol. Enzyme purified in the presence of pyridoxal-P and β-mercaptoethanol shows no activation. Enzyme purified in the presence of a thiol compound slowly loses activity when stored in the frozen state. After 6 to 8 months, when the activity has fallen to about 60% of its original value, the activity stabilizes for at least an additional 6 months. The addition of either pyridoxal-P or dithiothreitol partially restores the lost activity. The original activity can be restored only by incubating the enzyme with both pyridoxal-P and dithiothreitol.

The apoenzyme is converted to the oxidized form much more rapidly than is the holoenzyme. After 12 hours at 0° the apoenzyme loses its ability to be completely reactivated by pyridoxal-P only. After conversion of these apoenzyme preparations to holoenzyme by incubation with pyridoxal-P they exhibit the properties of the oxidized enzyme as described in the preceding paragraph.

Dissociation constants for the substrates glycine, serine, and allothreonine are the same for the oxidized and reduced enzymes. The dissociation constant of pyridoxal-P and the oxidized enzyme is 1.0×10^{-7} M^{-1} while for the reduced enzyme it is 6×10^{-8} M^{-1}. The pyridoxal-P content of the reduced and oxidized enzymes is the same as determined by either the phenylhydrazine method (16) or the cysteine method (1). Also, the two enzymes exhibit identical spectra.

The above data could also be explained by the presence of two enzymes in our purified preparation. One enzyme could be insensitive to thiol compounds whereas the other enzyme could be completely inactivated in the absence of a thiol compound. We have no evidence that our preparations contain two enzymes. If two enzymes were present they would be expected to be denatured at different rates at a high temperature. In Table II are recorded the results of an experiment where oxidized holoenzyme is incubated at 60°. At several time intervals aliquots were removed and assayed in the presence and absence of pyridoxal-P and dithiothreitol. The data show that the ratio of activities for the two assay procedures remains constant, within the limits of the accuracy of the assay, during 93% denaturation of the enzyme. This supports the view that a single enzyme is present in our preparations.

Titration of —SH Groups—We found that DTNB rapidly and completely inactivated the reduced and oxidized enzyme. To determine the number of —SH groups present, we titrated the enzyme with DTNB. The extent of the reaction was determined by measuring the increase in absorbance at 412 nm (1%). After each addition of DTNB, a 0.02-ml aliquot was removed and assayed for enzymatic activity. Fig. 3 shows that there are 12 reactive —SH groups per molecule of reduced enzyme or three —SH groups per monomer. When oxidized enzyme was titrated with DTNB only 8.0 —SH groups per molecule were found. The data in Fig. 3 also show that enzyme activity decreased more rapidly than the —SH groups. Reaction of one-third of the —SH groups resulted in a 50% loss in enzyme activity. This could be due to a difference in reactivity of the —SH groups with those required for enzyme activity being more reactive toward DTNB. We tested this by following the course of the reaction when an excess of DTNB was added. The reaction was performed at 11° so that the reaction could be followed with a recording spectrophotometer. Fig. 4 is a first order graphical analysis of this experiment and suggests that the —SH groups can be described as two classes. The data show that 7.7 —SH groups react rapidly with DTNB and 3.8 —SH groups react more slowly. We interpret this to mean that of the three —SH groups on each monomer, two react rapidly with DTNB, resulting in loss of enzymatic activity, while one —SH reacts more slowly. When this kinetic study was performed with the substrates glycine and serine present in the reaction mixture, no difference of rate or extent of reaction with DTNB was observable. When the DTNB-treated enzyme was dialyzed to remove the excess DTNB, we found that the 430-nm peak, due to the bound pyridoxal-P, had disappeared. This suggests that the reaction with DTNB had caused the pyridoxal-P to dissociate from the enzyme. Treatment of the DTNB enzyme with β-mercaptoethanol and pyridoxal-P restored 25% of the original activity.

A kinetic study of the reaction of the oxidized enzyme with DTNB reveals that of the eight —SH groups, four react rapidly and four react more slowly. This suggests that of the two —SH

TABLE II

Course of thermal denaturation of oxidized serine transhydroxymethylase

The oxidized enzyme used in this experiment had been stored at −15° for 8 months and contained eight DTNB-titratable —SH groups. A 1.7 mg per ml solution of enzyme in 0.01 M phosphate buffer, pH 7.2, was incubated at 60°. At several time intervals 10-μl aliquots were removed to determine the activity of the enzyme in the absence and presence of 1×10^{-4} M pyridoxal-P and 1×10^{-3} M dithiothreitol.

Time of incubation at 60°	Units of enzyme activity per 10 μl of reaction mixture		Ratio of activities without and with pyridoxal-P and dithiothreitol
	Without pyridoxal-P and dithiothreitol	With pyridoxal-P and dithiothreitol	
min			
0	76	124	0.61
30	42	69	0.60
70	27	42	0.61
130	14.5	22.5	0.64
250	6.2	9.5	0.65

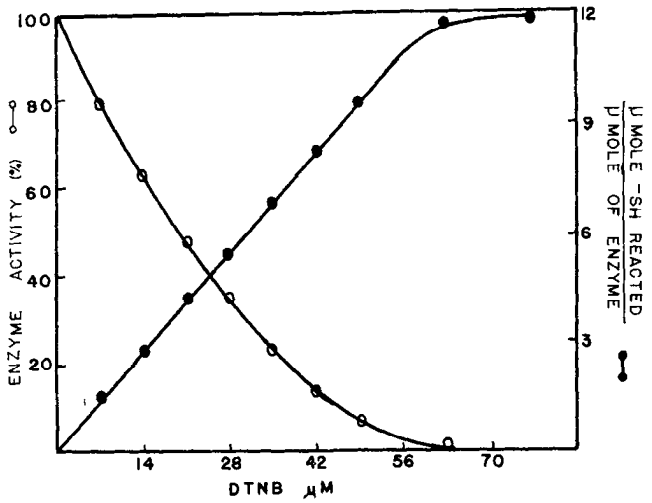

FIG. 3. Titration of —SH groups of serine transhydroxymethylase with DTNB. To 1.1 mg of enzyme in 1.0 ml of 0.05 M potassium phosphate, pH 7.3, were added 0.003-ml aliquots of a 2×10^{-3} M solution of the DTNB reagent. The reaction was followed by determining the increase in absorbance at 412 nm. When there was no further increase in absorbance at 412 nm a 0.02-ml aliquot was removed and tested for enzymatic activity in the allothreonine assay. The graph compares the number of —SH groups reacted per mole of enzyme, ●——●, and the enzymatic activity, ○——○, at various concentrations of DTNB.

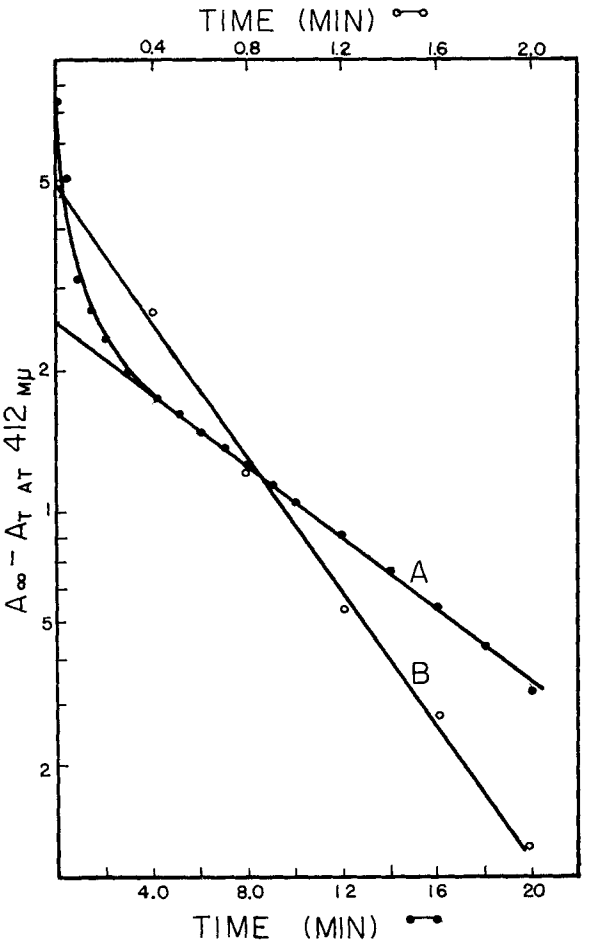

FIG. 4. Graphical analysis of the reaction of DTNB with serine transhydroxymethylase. To 0.97 mg of enzyme in 1.0 ml of 0.05 M potassium phosphate, pH 7.3, was added 0.5 μmole of DTNB. The reaction was followed by recording the rate of increase in absorbance at 412 nm (17). The reaction was run at 11°. *Curve A*, this is a semilogarithmic plot of the absorbance change at 412 nm ($A_{412\infty}$:$A_{412 \text{ at time } t}$) *versus* time. The straight line portion of this biphasic curve was used to calculate the number of slow reacting —SH groups. The *y* axis intercept of the extrapolated portion of the straight line shows an absorbance change of 0.250 which corresponds to the reaction of 3.8 —SH groups. *Curve B*, this line was obtained by subtracting the extrapolated line from *Curve A* from the corresponding values of the experimental portion of *Curve A* during the first 2 min of the reaction. The linear nature of *Curve B* indicates first order kinetics for the fast reacting —SH groups. The number of fast reacting —SH groups was calculated from the total absorbance change, 0.750, minus the absorbance change due to the slow reacting —SH groups, 0.250. This gives a value of 7.7 —SH groups for *Curve B*.

groups on each monomer that react rapidly with DTNB in the reduced enzyme, only one is available to DTNB in the oxidized enzyme. Blocking this one —SH group with DTNB causes complete loss of activity.

The reaction of apoenzyme with DTNB reveals the presence of eight —SH groups but they react so rapidly a kinetic analysis could not be done. This suggests that in the apoenzyme the —SH groups are more exposed than in the holoenzyme.

DISCUSSION

Previous investigations have shown that serine transhydroxymethylase consists of four subunits (3). This interpretation was based on the molecular weight of the native enzyme compared to the molecular weight of the dissociated enzyme in sodium dodecyl sulfate and the observation that 4 moles of pyridoxal-P are bound per mole of enzyme. In this paper we conclude that all four subunits are identical. This interpretation is supported by the peptide map analysis, the COOH-terminal amino acid studies, and the single band found in the disc electrophoresis experiments. These studies also indicate that our preparations are not a mixture of two isozymes reported to exist in rabbit liver (4).

The involvement of —SH groups in enzyme activation has been indicated for several pyridoxal-P enzymes (18). However, evidence for their participation in the mechanism of the reaction is not evident in most of these enzymes. The data in this paper would also fit this pattern. Although we can correlate the oxidation of four —SH groups with a change in V_{max} for the enzyme we have been unable to detect any other changes. The inactivation of the enzyme by DTNB is also a characteristic of many other pyridoxal-P enzymes (18). Since pyridoxal-P is lost from the DTNB-reacted enzyme the inactivity could be due to a loss of affinity of the enzyme for pyridoxal-P rather than showing that a —SH group is involved in the mechanism of the reaction.

The data in this paper explain why our preparations of serine transhydroxymethylase exhibit activation upon the addition of pyridoxal-P and a sulfhydryl compound. First, we have observed that high concentrations of $(NH_4)_2SO_4$ cause dissociation of pyridoxal-P from the enzyme. If cysteine is added to the solution to trap the pyridoxal-P as the thiazolidine complex total resolution of the enzyme can be achieved. Each step in the purification in which $(NH_4)_2SO_4$ is used results in partial resolution of the enzyme. Because the resolved enzyme is readily oxidized to the less active form of the enzyme, the oxidized form is accumulated at almost every step in the purification procedure. We originally stated erroneously that serine transhydroxymethylase was activated by pridoxal-P alone (1). What we did not realize at that time was that the β-mercaptoethanol, which was added to the assay to protect the required coenzyme tetrahydrofolate, was converting the oxidized enzyme to the reduced form.

The requirement of pyridoxal-P in addition to a thiol compound to reactivate the oxidized enzyme to the reduced form suggests that the oxidized form is not fully saturated with pyridoxal-P. The larger dissociation constant for the oxidized enzyme would support this interpretation. However, the identical spectra of the two enzyme forms and the similar values of pyridoxal-P content determined by two different chemical methods suggest that the oxidized enzyme is fully saturated. A more detailed study of the mechanism of the reaction of pyridoxal-P with the oxidized and reduced forms of the enzyme may resolve this question.

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