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INTERACTION OF PYRIDOXAL 5-PHOSPHATE WITH APO-SERINE HYDROXYMETHYLTRANSFERASE

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Summary

The interaction of pyridoxal 5-phosphate with beef liver serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1) has been investigated using sedimentation velocity, kinetic and equilibrium techniques. No evidence for an aggregating system could be found in sedimentation velocity experiments in the presence or absence of pyridoxal 5-phosphate. Reassociation of pyridoxal 5-phosphate with apoenzyme and reacquisition of enzymic activity follow identical kinetics. An initial fast step is followed by a second order process with a rate constant of 66 $M^{-1} \cdot s^{-1}$. A dissociation constant of 27.5 μM was obtained from equilibrium studies. No interaction of binding sites was exposed by altering pH or in the presence of glycine or folate. Maxima observed in pH profiles with both binding and reactivation are interpreted as the composite of two overlapping processes, one of which is ionization of the pyridinium nitrogen of pyridoxal 5-phosphate and the other a functional group on the apoenzyme. Evidence is presented to indicate the necessity for the formation of an enzyme · pyridoxal 5-phosphate Schiff's base complex during catalytic turnover.

Introduction

The mechanism of action of serine hydroxymethyltransferase (5,10-methylene tetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1) has been studied by steady-state [1—5] and transient [6—8] kinetics, equilibrium binding [5,9] and isotope exchange [5]. Several such studies have taken advantage of the spectral characteristics of enzyme bound pyridoxal 5-phosphate as a structural probe to better understand the nature of the catalytic site and catalytic turnover. Schirch and coworkers [10] have prepared apo-serine hydroxymethyltransferase from the rabbit liver enzyme. We have used both kinetic and equi-

librium techniques to follow the reassociation of pyridoxal 5-phosphate with beef liver apo-serine hydroxymethyltransferase. The results are interpreted in terms of elucidation of the nature of the functional catalytic site and the requirement for a Schiff's base bond between pyridoxal 5-phosphate and enzymic lysine as a necessary intermediate during catalytic turnover.

Materials and Methods

The L-cysteine, folic acid, 2-mercaptoethanol, and pyridoxal 5-phosphate were obtained from Sigma Chemical Company. Glycine and potassium phosphate were obtained from Fisher Scientific Company and $(NH_4)_2SO_4$ from Schwarz-Mann.

A Beckman Acta CIII UV-visible spectrophotometer was used in the double beam mode for the spectral studies. The cell chamber was thermostatically maintained at 25°C by circulating water. Routine assays for enzymic activity with DL-threo-β-phenylserine as substrate were carried out as previously described [1].

Beef liver serine hydroxymethyltransferase was purified as previously reported [1] or by a procedure which Schirch and Gross [11] developed for the rabbit liver enzyme. Preparations were routinely greater than 90% pure based on Coomassie Blue staining of electrophoretic SDS polyacrylamide gels.

The apoenzyme was prepared by a modification of the procedure used by Schirch et al. [10] for the rabbit liver enzyme. Solid L-cysteine was added to enzyme solution (4—5 mg/ml) to produce a 0.1 M solution. The yellow color of the holoenzyme immediately began to fade and, after several minutes, solid $(NH_4)_2SO_4$ was added to 30% saturation. The resulting solution was dialyzed for 12 h against two changes of 25 mM potassium phosphate (pH 7.5)/2 mM 2-mercaptoethanol/0.1 M L-cysteine/30% $(NH_4)_2SO_4$. The apoenzyme was then dialyzed exhaustively against 25 mM potassium phosphate (pH 7.5)/2 mM 2-mercaptoethanol prior to use.

Sedimentation velocity experiments were performed at room temperature in a Beckman Model E analytical ultracentrifuge equipped with Schlieren optics. 6.0 mg/ml apo or holo-serine hydroxymethyltransferase in 25 mM potassium phosphate (pH 7.5)/2 mM 2-mercaptoethanol were employed.

Enzyme reactivation kinetics were followed by assaying for β-phenylserine aldolase activity after the addition of pyridoxal 5-phosphate to solutions of apo-serine hydroxymethyltransferase. Reassociation of pyridoxal 5-phosphate with the apoenzyme was followed by monitoring the increase in absorbance at 430 nm associated with formation of the azomethine linkage between pyridoxal 5-phosphate and an enzymic lysine residue.

Results

Beef liver serine hydroxymethyltransferase was sedimented in an analytical ultracentrifuge in the presence and absence of pyridoxal 5-phosphate. Holoenzyme preparations consist predominantly of a species sedimenting at an observed 9 S. The apo-enzyme sediments with an indistinguishable s value. Both also contain a minor species sedimenting at 6 S. No difference in the relative

quantity of the major and minor components could be detected between the apo- and holoenzyme. Further, no new band or any suggestion of a reduction in the total quantity of protein was observed. The subunit aggregation states thus appear identical for the apo and holoenzymes.

A visible absorption band at 430 nm in preparations of the holoenzyme can be attributed to a Schiff's base complex between pyridoxal 5-phosphate and an ϵ-amino group of lysine [15]. In Fig. 1 it can be seen that the kinetics of the reacquisition of the 430-nm band upon addition of pyridoxal 5-phosphate to apo-serine transhydroxymethylase involves at least two processes. The first process, suggested by the non-zero intercept, is too fast to be monitored under the conditions used. Subsequent to the first process, the entire time course of the reacquisition of the 430 nm band is adequately described as second order, with a rate constant estimate of 66 $M^{-1} \cdot s^{-1}$. Reacquisition of β-phenylserine aldolase activity is also shown in Fig. 1. It can be seen that the two processes are kinetically identical.

The fractional reactivation as well as the reacquisition of the 430 nm band can be interpreted in terms of establishment of equilibrium if reactions are allowed to proceed to completion. An estimate of the dissociation constant for pyridoxal 5-phosphate binding to the apoenzyme can thus be obtained. In Fig. 2 is shown an essentially linear reciprocal plot derived from reacquisition of activity. Reacquisition of the 430 nm absorption band yielded an essentially identical plot. A dissociation constant, K_d, of 27.5 μM is obtained if independent subunits of 55 000 daltons are assumed [14].

To describe the reassociation of pyridoxal 5-phosphate to apo-serine hydroxymethyltransferase more fully, the pH dependence of the process was deter-

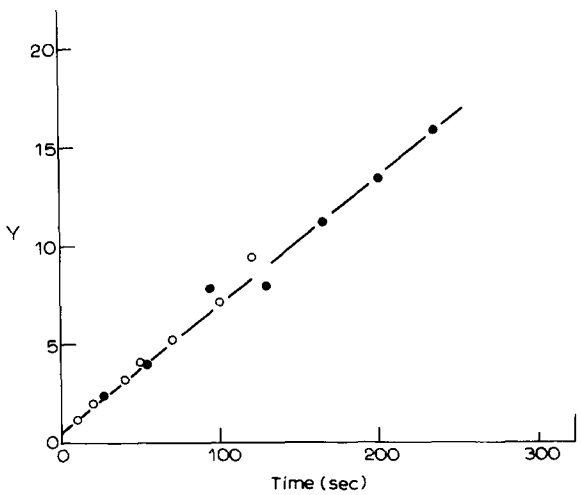
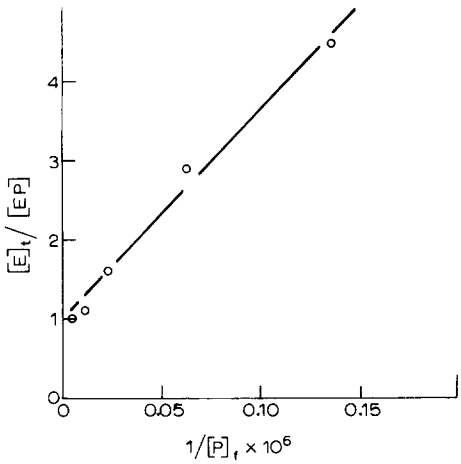


Fig. 1. Acquisition of 430 nm absorption band following reassociation of pyridoxal 5-phosphate with apo-serine hydroxymethyltransferase (○) and recovery of β-phenylserine aldolase activity (●) as function of time. The ordinate (Y) represents $1/(P_o - E_o)$ ln $[E_o(P_o - x)/P_o(E_o - x)]$, where P_o is initial pyridoxal 5-phosphate concentration, E_o is initial apoenzyme concentration (determined as μmoles subunit/l) and x is fraction of apoenzyme converted to holoenzyme at any given time. Initial apoenzyme concentration (based on a subunit molecular weight of 55 000) was 8.7 μM and initial pyridoxal 5-phosphate concentration was 25 μM.



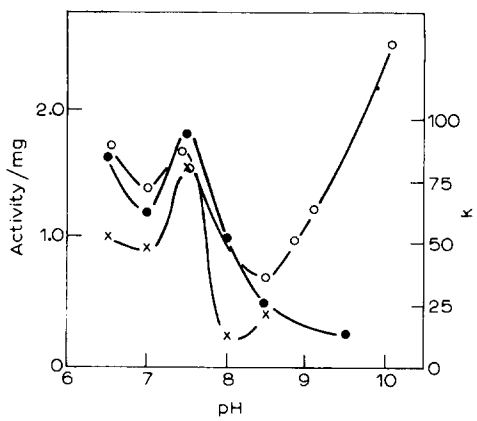


Fig. 2. Reciprocal of maximum degree of reacquisition of β-phenylserine aldolase activity ($[E]_t/[EP]$) as a function of reciprocal pyridoxal 5-phosphate concentration. $[E]_t$ is total apoenzyme concentration (8.7 μM) and [EP] is the concentration of holoenzyme. An equilibrium constant was estimated from the equation, $[E]_t/[EP] = K_d/[P]_f + 1$.

Fig. 3. pH profiles for maximum activity recovered (X), second-order rate constants for reacquisition of β-phenylserine aldolase activity (●) and second-order rate constants for reacquisition of 430 nm absorption band (○). Initial apoenzyme concentration was 8.4 μmol subunit/l and initial pyridoxal 5-phosphate concentration was 25 μM.

mined. In Fig. 3 it can be seen that the maximum activity which can be recovered, the rate constants for reactivation of β-phenylserine aldolase activity, and the rate constants for reacquisition of the 430 nm absorption band all exhibit maxima in the pH range from 7.4—7.8.

In contrast to the marked effects caused by changes in solution pH, the presence of folate or glycine had no apparent effect on the kinetics of reacquisition of β-phenylserine aldolase activity when apoenzyme was preincubated with pyridoxal 5-phosphate in their presence (Fig. 4). The presence of folate causes a greater final level of activity to be attained, consistent with the activation by this component previously reported [1]. Glycine, on the other hand, a

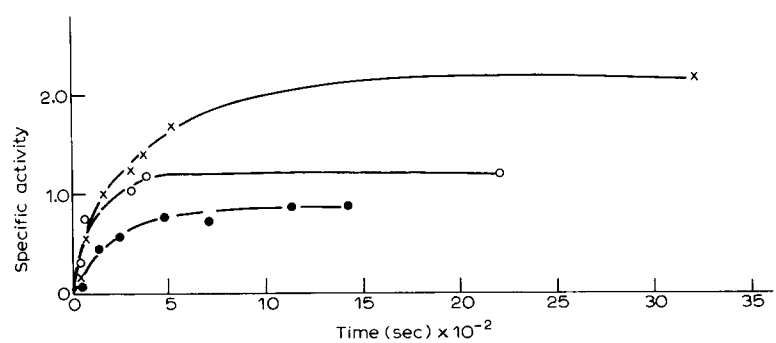


Fig. 4. Reacquisition of β-phenylserine aldolase activity as a function of time in the presence of 500 μM folate (X), 0.48 M glycine (●), and in the absence of added effector or substrate (○). Initial apoenzyme and pyridoxal 5-phosphate concentrations were the same as those in Fig. 2.

product inhibitor of the β-phenylserine aldolase reaction [2], causes a diminution of the final specific activity. Both processes, however, can be described with the same second order rate constant as in their absence.

Discussion

Activation of the β-phenylserine aldolase activity of serine hydroxymethyltransferase by folate has been interpreted in terms of enhanced substrate binding caused by the presence of folate at the catalytic site [1]. Allosteric or site-site interaction mechanisms could also be used to explain the same phenomenon since they are redundant with random binding mechanisms so far as steady-state kinetics are concerned [12,13]. Tryptophanase, another tetrameric, pyridoxal 5-phosphate containing enzyme, has been shown to dissociate into dimers and monomers in the apo- state but not in the holo- state [16]. Tetrameric serine hydroxymethyltransferase [14] could also undergo subunit aggregation mediated by pyridoxal 5-phosphate and such a mechanism could be used to explain the observed steady-state kinetics [1]. No evidence, however, could be found for a dissociating system in sedimentation velocity experiments regardless of the presence or absence of pyridoxal 5-phosphate.

Since other types of subunit interaction mechanisms which do not entail changes in aggregation state could also explain the observed kinetics [1] of serine hydroxymethyltransferase [17,18], it was necessary to rule them out as well. Using both kinetic and equilibrium techniques and the sensitive internal probe, pyridoxal 5-phosphate, we have been unable to obtain any evidence of interaction between prosthetic group binding sites. Since these studies were conducted as a function of pH under conditions which significantly altered both rates and equilibria, redundancy in sites which may have been present at a single pH should have been removed. However, no new binding sites could be identified. Further, the presence of the substrate, glycine, or effector, folate did not expose or give rise to evidence for interaction between binding sites of serine hydroxymethyltransferase.

It can be assumed that there is significant bonding between pyridoxal 5-phosphate and serine hydroxymethyltransferase other than through the lysyl Schiff's base bond, since dissociation of the pyridoxal 5-phosphate complex does not occur upon the addition of glycine. The amino function of glycine displaces the ε-amino group of enzymic lysine in this reaction. Further, during catalytic turnover, the amino acid substrate (eg. serine) must enter into a Schiff's base bond with pyridoxal 5-phosphate, yet the complex apparently does not dissociate significantly. Thus, pyridoxal 5-phosphate can reside on the enzyme without the benefit of bonding contributed by the azomethine linkage to the lysyl residue. This raises the question whether or not such an enzymic lysyl azomethine bond is formed as a necessary intermediate during catalysis. The free aldehydic group of pyridoxal 5-phosphate can occur after product release and the aldehyde moiety can then act as an acceptor for the next incoming amino acid. However, the correspondence between reacquisition of the 430 nm absorption band and the reacquisition of full activity strongly suggests the requirement for a lysyl-azomethine bond as a necessary catalytic intermediate. This would suggest that one reaction on the pathway required for

turnover is displacement of the enzymic lysine by the incoming amino acid moiety.

Although free pyridoxal 5-phosphate has a pK_a of 8.7 [19], the pK_a for the glycyl-serine hydroxymethyltransferase complex occurs at 6.9 [2]. Reacquisition of both the 430 nm absorption band and enzymic activity exhibit maxima at an intermediate pH (approx. 7.4) but closer to the pK_a of the glycyl-enzyme complex. While it is typical for the sum of the complex processes involved in enzymic activity to exhibit a pH maximum, it is more difficult to explain the maxima observed for the binding and Schiff's base formation between pyridoxal 5-phosphate and apo-serine hydroxymethyltransferase. It appears that at least two pH dependent processes are required to explain such phenomena. By analogy to the glycyl · enzyme complex dissociation results, one of these processes probably involves deprotonation of the pyridinium nitrogen from the β-phenylserine · enzyme complex in the active site environment. The second process is presumably related to a functional group residing on the apoenzyme. The precise identification of such a functional group will be the subject of future studies.

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