Transient Kinetic Studies on the Allosteric Transition of Phosphoglycerate Dehydrogenase*

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Stopped flow spectrophotometry was used to investigate the kinetics of the transition of the phosphoglycerate dehydrogenase (3-phosphoglycerate: NAD oxidoreductase, EC 1.1.1.95) reaction from the active to the inhibited rate upon the addition of the physiological inhibitor serine. The transition was characterized by a single first order rate constant ($k_{on,i}$) which was independent of enzyme concentration. At pH 8.5, $k_{on,i}$ increased in a hyperbolic manner with serine concentration from 2 to 8 s$^{-1}$. The increase in $k_{on,i}$ occurred at serine concentrations where the steady state inhibition was virtually complete. These results indicate that serine inhibition is an allosteric process involving a conformational change in the enzyme. A model is presented in which serine at low concentrations binds exclusively to the inhibited state of the enzyme and shifts the equilibrium toward that state; at high serine concentrations, serine binds to the active state, facilitating its conversion to the inhibited state. An alternative model, which we favor, proposes two classes of inhibitor binding sites.

The kinetics of the fluorescence quenching of enzyme-bound NADH by serine (Sugimoto, E., and Pizer, L. I. (1968) J. Biol. Chem. 243, 2090-2098), measured by stopped flow fluorimetry, was also characterized by a single first order rate constant ($k_{obs,i}$) which was independent of enzyme concentration. At pH 8.5, $k_{obs,i}$ ranged from 0.4 s$^{-1}$ at low serine concentrations to 1.1 s$^{-1}$ at high serine concentrations. These results indicate that the fluorescence quenching induced by serine is a manifestation of a structural change in the enzyme.

Enzyme and excess NADH were mixed with substrate and serine in the stopped flow instrument, and enzyme-bound NADH fluorescence was monitored by exciting through the protein at 285 nm. A rapid fluorescence quenching process, which occurred within the mixing time, was followed by a slower fluorescence enhancement process which terminated in a steady state level corresponding to the quenched fluorescence of the enzyme-NADH-serine complex. The rapid quenching was the result of substrate binding (Dubrow, R., and Pizer, L. I. (1977) J. Biol. Chem. 252, 1539-1551). The fluorescence enhancement was characterized by a single first order rate constant whose value for a given serine concentration corresponded with $k_{obs,i}$. This data shows that the quenched state of the enzyme-NADH complex is the state which is directly responsible for the inhibition of enzyme activity. During catalysis the quenched state is achieved from a different initial conformation, and consequently at a different rate, than in the absence of substrate.

$k_{on,i}$ and $k_{obs,i}$ were also measured using glycine, another inhibitor. The ultraviolet difference spectrum between enzyme and enzyme plus serine was determined and proposed to be the result of the same structural change which is responsible for the fluorescence quenching by serine.

Much effort has gone into obtaining an understanding at the molecular level of the interaction between small molecular weight effectors and enzymes. The concept of allosteric regulation was introduced by Monod et al. (1). Generalizing from information which had accumulated about several bacterial regulatory enzymes, they postulated the existence of two distinct specific sites on these enzymes: the active site, or catalytic site, and the allosteric site, the site to which the effector molecule was bound. Binding of the effector at the allosteric site mediates a reversible alteration (allosteric transition) in the structure of the protein, which modifies the properties of the active site. A further conceptual advancement followed from the observation that most regulatory enzymes are oligomers containing identical subunits. Cooperative interaction between substrate or effector binding sites accounted for the sigmoidal shaped saturation or inhibition curves which are characteristic of regulatory enzymes. Detailed mechanistic models of allosteric regulation have been presented (2, 3).

Because of the universal character of allosteric regulation and its importance in metabolic regulation, detailed mechanistic studies appear warranted. Conclusions drawn from one system may contribute in a general sense to our understanding of allosteric regulation. This report describes the investigation of phosphoglycerate dehydrogenase (3-phosphoglycerate:NAD oxidoreductase, EC 1.1.1.95) from Escherichia coli. This enzyme catalyzes the reaction: 3-phosphoglycerate + NAD$^+$ + H_2O $\rightarrow$ hydroxypyruvate-P + NADH + H$^+$. Phosphoglycerate dehydrogenase, the first enzyme in the serine biosynthetic pathway, is inhibited by serine (4). This feedback loop constitutes the major mode of regulation of serine biosynthesis in E. coli (5, 6). Phosphoglycerate dehydrogenase has been purified to homogeneity, and many of its physical, chemical, kinetic,
and optical properties have been described (7-12). It is a tetramer of molecular weight 163,000 (9) containing identical subunits (10). There are four coenzyme binding sites per enzyme molecule (8). The number of serine binding sites has not been unambiguously determined.

Sugimoto and Pizer (7, 8) have proposed that serine inhibits phosphoglycerate dehydrogenase via an allosteric mechanism based on: (a) the basically noncompetitive character of serine inhibition with respect to substrates and coenzymes; (b) the sigmoid shape of the serine inhibition curve; and (c) the quenching of the fluorescence of enzyme-bound NADH by serine, which was interpreted as being a manifestation of a conformational change in the enzyme. This evidence, along with the absence of both homotropic effects for the substrates and coenzymes and heterotropic effects, indicated that phosphoglycerate dehydrogenase might be classified as a V-type allosteric enzyme (2). This report describes results from stopped flow measurements, modeled after those performed with homoserine dehydrogenase (13, 14), on the kinetics of changes in enzyme activity and enzyme-bound NADH fluorescence caused by serine and glycine, another inhibitor. These results provide further insight into the allosteric properties of phosphoglycerate dehydrogenase.

**MATERIALS AND METHODS**

**Reagents**—Serine and glycine were obtained from Calbiochem (A grade). The glycine was recrystallized twice from hot water to ensure that it was free from traces of serine. The sources of other reagents are provided in the accompanying paper (15).

**Phosphoglycerate Dehydrogenase**—The purification and storage of phosphoglycerate dehydrogenase are described in the accompanying paper (15). For use in an experiment, the purified enzyme was passed through a small Sephadex G-25 column equilibrated with the proper buffer. The enzyme concentration was estimated from the absorbance at 260 nm (8).

**Kinetic Measurements**—Rapid absorbance or fluorescence changes were measured using a Durrum-Gibson stopped-flow spectrophotometer equipped with a fluorescence attachment. Spectrophotometric measurements were made by following the percentage of transmission at 340 nm as a function of time. The light source was either a 75-watt xenon lamp (Osram or Illumination Industries) or a tungsten lamp. The photomultiplier detected the fluorescence at right angles to the exciting beam.

In the fluorescence experiments, enzyme-bound NADH was excited either directly at 340 nm or through the protein at 295 nm. The fluorescence was observed using a 400 to 3000 nm or a 450 to 3000 nm secondary filter. A 75-watt xenon lamp was used as the light source. The photomultiplier detected the fluorescence at right angles to the exciting beam, with the photomultiplier voltage adjusted to the appropriate sensitivity. A photometric log amplifier was used in some of the experiments.

All stopped flow determinations were thermostatted at 25° ± 0.5°. The mixing time was less than 4 ms. An adequate time constant (<5% of the oscilloscope time base setting) was used.

Rate constants were calculated from photographs of the oscilloscope tracings. In all experiments, equal volumes (~0.15 ml) of reactants were mixed. Concentrations given in the text are the concentrations obtained after mixing unless otherwise stated. The estimated error in the determination of rates and amplitudes for the stopped flow experiments is ±16%.

**RESULTS**

**Kinetics of Transition of Enzyme Activity from Active to Inhibited Rate**—The following terminology will be used. The pre-steady state portion of the inhibition process is the period between the addition of a given quantity of serine to phosphoglycerate dehydrogenase, which is turning over at its fully active rate, and the achievement of the final zero order inhibited rate caused by that quantity of serine. The final inhibited rate is the steady state level of inhibition.

The steady state level of inhibition of the reverse direction reaction (hydroxypyruvate-P reduction) as a function of serine concentration at the two pH values used in these studies (7.5 and 8.5) was determined (Fig. 1). The reverse direction was assayed because the higher turnover number in this direction made the subsequent stopped flow experiments technically easier to perform. The enzyme assays were done in the presence of saturating concentrations of NADH and hydroxypyruvate-P. There was a sigmoidal dependence of the extent of inhibition on the concentration of serine at both pH values.

The Hill number (n1), which is a quantitative measure of sigmoidicity, was 2.0 at pH 7.5 and 2.4 at pH 8.5. The quantity of serine which produced a 50% level of inhibition (I50) was 4 μM at pH 7.5 and 5.1 μM at pH 8.5. The Hill numbers and I50 values varied slightly among enzyme preparations (e.g. n = 1.96 ± 0.32, I50 = 4.1 ± 0.3 μM at pH 7.5). In all preparations, greater than 99% inhibition was achieved at high serine concentrations. Thus small differences in subunit interactions, rather than desensitization of a portion of the enzyme to serine, is the likely explanation for the variation.

The pre-steady state portion of the inhibition process was observed using a stopped flow spectrophotometer. Fig. 2 shows photographs of the original oscilloscope traces of this experiment along with two controls. Fig. 2B represents the experiment in which phosphoglycerate dehydrogenase and NADH were mixed with hydroxypyruvate-P and 40 μM serine. After mixing, transmission at 340 nm was monitored as a function of time. NADH and hydroxypyruvate-P were present in saturating amounts after mixing. The transition of the enzyme reaction from the active to the inhibited state was observed. The initial rate was the rate of fully active enzyme. This rate gradually diminished until the steady state inhibited rate corresponding to 20 μM serine was achieved.

In the control experiment shown in Fig. 2A, enzyme and NADH were mixed with hydroxypyruvate-P. The initial zero order rate was the rate of fully active enzyme. The turnover number under these conditions was the same as the turnover number measured in the conventional spectrophotometric assay, in which a 50- to 100-fold lower enzyme concentration was used. Thus no complications were introduced by using the higher enzyme concentrations necessary for stopped flow measurements.

In the control experiment represented in Fig. 2C, phosphoglycerate dehydrogenase was preincubated with 40 μM serine. Enzyme, NADH, and serine were mixed with hydroxypyruvate-P. The initial zero order rate was 8% of the rate of fully active enzyme seen in Fig. 2A.

The same type of experiment done under different conditions of pH and serine concentration gave oscilloscope traces of the same general form as Fig. 2B.

These experiments were analyzed on the basis of a first order decay of active enzyme using equations presented by Barber and Bright (13). The function 1 - P(t), defined by these experiments on the basis of a first order decay of active enzyme using equations presented by Barber and Bright (13). The function 1 - P(t), defined by these
investigators, is equivalent to the fraction of active inhibitible enzyme remaining at time \( t \). The semilog plots of \( 1 - F(t) \) versus time were linear for the pre-steady state inhibition of phosphoglycerate dehydrogenase by serine under all conditions tested (e.g., Fig. 2B, inset). Thus the kinetics of the transition of the enzyme reaction from the active to the inhibited rate, for a given reaction condition, can be characterized by a single observed first order rate constant \( (k_{\text{obs},i}) \).

When the serine concentration was held constant, \( k_{\text{obs},i} \) was independent of enzyme concentration at both pH 7.5 and 8.5. The dependence of \( k_{\text{obs},i} \) on substrate and coenzyme concentration was examined at pH 8.5. For a given serine concentration, \( k_{\text{obs},i} \) was independent of NADH concentration over a 4-fold range from 25 to 100 \( \mu \text{M} \) (Fig. 3), and was independent of hydroxypyruvate-P concentration from 47 to 375 \( \mu \text{M} \) (Fig. 3).

The dependence of \( k_{\text{obs},i} \) on the concentration of serine is shown in Fig. 3. At pH 7.5, varying the serine concentration had little effect on \( k_{\text{obs},i} \). Over a 100-fold range in serine concentration, \( k_{\text{obs},i} \) increased from 1 to 1.5 s\(^{-1}\). The dependence of \( k_{\text{obs},i} \) showed a different pattern at pH 8.5. Between 10 and 100 \( \mu \text{M} \) serine, \( k_{\text{obs},i} \) increased from 2 to 8 s\(^{-1}\). Between 10\(^{-4}\) and 10\(^{-3}\) \( \mu \text{M} \) serine, \( k_{\text{obs},i} \) remained 8 s\(^{-1}\). The serine concentration at which 50% of the increase in \( k_{\text{obs},i} \) had occurred was 55 \( \mu \text{M} \).

All of these experiments were performed at serine concentrations which cause greater than 70% steady state inhibition. Lower serine concentrations could not be used because of difficulty in evaluating \( k_{\text{obs},i} \) when there were only small differences between the rate of fully active enzyme and the steady state inhibited rate. It should be noted that the increase in \( k_{\text{obs},i} \) as a function of serine concentration at pH 8.5 occurred at serine concentrations where the steady state inhibition was virtually complete. This difference between the serine dependence of the steady state inhibition (Fig. 1) and the serine dependence of \( k_{\text{obs},i} \) at pH 8.5 must be taken into account in any model designed to explain the data.

We conclude from the first order nature of the pre-steady state inhibition kinetics and the serine and enzyme concentration dependencies of \( k_{\text{obs},i} \) that serine inhibition of phosphoglycerate dehydrogenase is an allosteric process (see "Discussion").

Kinetics of Quenching of Enzyme-bound Reduced Pyridine Nucleotide Fluorescence by Serine—If a serine-induced conformational change in the enzyme is responsible for the inhibition of enzyme activity, the kinetics of that change should
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FIG. 3. Dependence of $k_{\text{obs},j}$ on serine concentration. Experiments such as those described in Fig. 2B were performed. At pH 8.5, two different enzyme concentrations (0.5 μM and 1 μM) and three different NADH concentrations (●, 25 μM; ▲, 50 μM; ■, 100 μM) were used in obtaining the data. The initial experiments were done using 100 μM NADH. The NADH concentration was lowered for subsequent experiments because for a given change in concentration, the change in transmission was greater, the lower the initial concentration. Thus the quality of the experiments was improved. At pH 7.5, 0.4 μM enzyme and 20 μM NADH were used. All other conditions were the same as those described in Fig. 2B except that the serine concentration was varied. $k_{\text{obs},j}$ (closed symbols) was evaluated from semilog plots such as those shown in Fig. 2B (inset). The open circles represent the final steady state level of inhibition achieved in these experiments. The steady state inhibited rates were obtained from the same traces as the $k_{\text{obs},j}$ values, and compared to the rate of fully active enzyme, which was determined in a control experiment.

correlate with the kinetics of the transition of the enzyme reaction from the active to the inhibited rate. Sugimoto and Piser (8) demonstrated in a static experiment that serine quenched the fluorescence of phosphoglycerate dehydrogenase-bound NADH and postulated that the fluorescence quenching was due to a conformational change in the enzyme. The experiments presented in this section were designed to test whether the kinetics of the fluorescence change were consistent with that hypothesis, and whether the kinetics correlated with the kinetics of the loss of enzyme activity. The experiments were performed in a stopped flow instrument using a fluorescence attachment. Phosphoglycerate dehydrogenase and NADH were mixed with serine and the fluorescence of enzyme-bound reduced pyridine nucleotide was observed. The quantity of NADH present after mixing was sufficient to effectively saturate the coenzyme binding sites as determined by fluorescence titrations.

Fig. 4 shows photographs of two oscilloscope tracings from representative experiments. Control experiments in which enzyme, NADH, and 1 mM serine contained in one syringe were mixed with buffer. No reaction was seen, and the fluorescence level was the final quenched level observed in the quenching reaction.

The kinetics of fluorescence quenching under all conditions tested was a single first order process. The insets in Fig. 4 show first order plots of the fraction of quenchable fluorescence remaining versus time derived from representative experiments. Observed first order rate constants ($k_{\text{obs},j}$) were obtained from these plots. When the serine concentration was held constant, $k_{\text{obs},j}$ did not vary significantly with enzyme concentration.

The dependence of $k_{\text{obs},j}$ on serine concentration is shown in Figs. 5 and 6. The amplitude of the decrease in fluorescence as a function of serine concentration is also shown. At pH 7.5, varying the serine concentration over a 1000-fold range (2 μM to 10 mM) had a minimal effect on $k_{\text{obs},j}$, which increased from 0.55 to 0.87 s⁻¹. At pH 8.5, $k_{\text{obs},j}$ (0.4 s⁻¹) was independent of serine concentration between 4 and 20 μM. $k_{\text{obs},j}$ then was not being measured. As an additional control, enzyme, NADH, and 1 mM serine contained in one syringe were mixed with buffer. No reaction was seen, and the fluorescence level was the final quenched level observed in the quenching reaction.
probably exists, as evidenced by the fact that 3-phosphoglycerate dehydrogenase is turning over, as in the pre-steady state inhibition experiments, it is in a different conformation (perhaps induced by the substrate, hydroxypyruvate-P) than it is as an enzyme-reduced coenzyme complex. The rate of achieving the quenched state from the "turning over" conformation could be faster than the rate of achieving the quenched state from the enzyme-reduced coenzyme complex conformation. According to this model, the quenched state could still be the conformation which is directly responsible for the inhibition.

This hypothesis was first tested by looking at the rate of fluorescence quenching by serine in the pre-steady state inhibition process. For a given serine concentration, the increase in $k_{ob,1/2}$ at pH 8.5 occurred at serine concentrations where the amplitude of the decrease in fluorescence was already at its maximum level. The serine concentration at which 50% of the increase in $k_{ob,1/2}$ had occurred was 100 $\mu$M, whereas the serine concentration at which 50% of the maximum decrease in amplitude had occurred was 10 $\mu$M.

These results are qualitatively similar to the results obtained for the pre-steady state kinetics of the inhibition process. They provide strong evidence that the fluorescence quenching by serine is the result of a conformational change in the enzyme. At pH 7.5, for a given serine concentration, the observed first order rate constants for the fluorescence quenching process were about $2/3$ to $1/2$ of the rate constants for the pre-steady state inhibition process. At pH 8.5, the discrepancy was larger. For a given serine concentration, there was a 5- to 7-fold difference between the rates of the two processes.

A possible explanation of this discrepancy is that when phosphoglycerate dehydrogenase is turning over, as in the pre-steady state inhibition experiments, it is in a different conformation (perhaps induced by the substrate, hydroxypyruvate-P) than it is as an enzyme-reduced coenzyme complex. The rate of achieving the quenched state from the "turning over" conformation could be faster than the rate of achieving the quenched state from the enzyme-reduced coenzyme complex conformation. According to this model, the quenched state could still be the conformation which is directly responsible for the inhibition.

This hypothesis was first tested by looking at the rate of fluorescence quenching in the presence of 3-phosphoglycerate, the product of the reverse direction reaction. It was felt that 3-phosphoglycerate would act as a substrate analogue. The abortive enzyme-NADH-3-phosphoglycerate ternary complex probably exists, as evidenced by the fact that 3-phosphoglycerate at the concentration used in this experiment, inhibits the initial velocity of the reverse direction reaction by 80%. By using 3-phosphoglycerate the problem of using hydroxypyruvate-P, which would oxidize the NADH reporter group, would be avoided. The results of this experiment are represented by the closed circles in Fig. 6. 3-Phosphoglycerate did not significantly alter the rate of fluorescence quenching.

A second difference, to be discussed later, between the kinetics of fluorescence quenching and the pre-steady state inhibition kinetics at pH 8.5 was the serine concentration at which 50% of the increase in the observed first order rate constant had occurred. It was 100 $\mu$M in the fluorescence quenching experiments and 55 $\mu$M in the pre-steady state inhibition experiments.

Kinetics of Enhancement by Serine of Enzyme-bound Reduced Pyridine Nucleotide Fluorescence during Pre-steady State Inhibition Process – In the experiments described in this section, the kinetics of the change in enzyme-bound NADH fluorescence caused by serine at pH 8.5 was examined under conditions identical with those used to monitor the pre-steady state inhibition kinetics, except that fluorescence was observed instead of transmission. The excitation was through...
the protein at 285 nm, and bound pyridine nucleotide fluorescence was observed through a 400 nm cut-off filter. Excitation was done through the protein, rather than directly, in order to reduce the background change in fluorescence due to oxidation of NADH in the reaction. (Serine quenches the fluorescence of the enzyme-NADH complex when it is excited through the protein in a similar manner when it is excited directly.)

The results show a rapid fluorescence quenching, that occurred within the mixed time (<4 ms), followed by a slower fluorescence enhancement process which terminated in a steady state level of fluorescence lower than the initial fluorescence of the phosphoglycerate dehydrogenase plus NADH (Fig. 7).

The top trace in Fig. 7 was a base-line control in which enzyme and NADH were mixed with buffer to determine the unperturbed fluorescence level of enzyme and NADH. The initial quenching was greater than 95% of the enzyme-bound reduced pyridine nucleotide fluorescence and is the result of hydroxypyruvate-P binding (discussed in accompanying paper (15)).

Two other controls were performed:

1. Serine was preincubated with enzyme. Phosphoglycerate dehydrogenase, NADH, and 1 mM serine were mixed with hydroxypyruvate-P. No reaction was seen, and the fluorescence level was the level to which the fluorescence was enhanced in the actual experiment (when the loss of fluorescence in the actual experiment due to the oxidation of NADH in the reaction was corrected for (see "Appendix")). The fluorescence level was also the same level as when enzyme, NADH, and 1 mM serine were mixed with buffer. Thus the fluorescence enhancement was a serine-dependent process, and the quenched fluorescence of the enzyme-NADH-serine complex was not altered by hydroxypyruvate-P. The final level of fluorescence achieved by the enhancement process was the same as the quenched fluorescence level of the enzyme-NADH-serine complex.

2. The reaction was run without serine (Fig. 8A). Enzyme and NADH were mixed with hydroxypyruvate-P. There was a lag of about 50 ms followed by a zero order decrease in fluorescence. The lag was shown not to be due to an inadequate instrumental time constant, and has been shown to be due to a rapid isomerization of the enzyme during the first turnover (see accompanying paper (15)). (A lag in the fluorescence enhancement process can also be seen (Fig. 8B). Its implications will be discussed later.) The zero order decrease in fluorescence was due to the disappearance of NADH in the reaction. Free NADH does fluoresce somewhat when activated at 285 nm. This control again demonstrates that the fluorescence enhancement is a serine-dependent process.

The results from these experiments could be analyzed on the basis of a single first order process (see "Appendix"). This is demonstrated by the linearity of the semilog plots of the fraction of enhanceable fluorescence remaining versus time (Fig. 7, inset). (The 50-ms lag, which will be explained later, was ignored in deriving these plots.) The observed first

![Fig. 7.](https://example.com/fig7.png)

**Fig. 7.** Enhancement of enzyme-bound NADH fluorescence during the pre-steady state inhibition process. Photograph of an original oscilloscope trace from a stopped flow experiment designed to measure the kinetics of the change in enzyme-bound NADH fluorescence caused by serine in the presence of NADH and hydroxypyruvate-P. The excitation wavelength was 285 nm. The top trace was a base-line control in which enzyme and NADH were mixed with buffer. This determined the unperturbed fluorescence level of enzyme and NADH. The bottom trace represents the experiment, in which enzyme and NADH were mixed with hydroxypyruvate-P and serine. A rapid quenching followed by a slower enhancement was observed. Reagent concentrations after mixing: 25 μM NADH, 375 μM hydroxypyruvate-P, 1 μM enzyme, and 1 mM serine. The inset shows representative semilog plots of the fraction of enhanceable fluorescence remaining versus time. Zero time for these plots was taken as 40 ms after mixing so that the lag was not included. ΔF/F₀ is the fraction of enhanceable fluorescence remaining.

![Fig. 8.](https://example.com/fig8.png)

**Fig. 8.** Enhancement of enzyme-bound NADH fluorescence during the pre-steady state inhibition process and the control in the absence of serine. Photographs of original oscilloscope traces from stopped flow experiments designed to measure the kinetics of the change in enzyme-bound NADH fluorescence caused by serine in the presence of NADH and hydroxypyruvate-P. The excitation wavelength was 285 nm. A, uninhibited reaction. Enzyme and NADH were mixed with hydroxypyruvate-P. B, enzyme and NADH were mixed with hydroxypyruvate-P and serine. The reagent concentrations after mixing were the same as in Fig. 7, except for the serine concentration, which was 100 μM. It should be noted that the time and millivolt scales were the same for the two traces.
order rate constants \( k_{obs,fr} \) for four different serine concentrations, along with the values of \( k_{obs} \) obtained for these serine concentrations, are presented in Table 1. The magnitudes of the two rate constants correlated well.

The fluorescence enhancement experiment was also carried out by exciting at 340 nm. There was an initial rapid quenching of 85% of the enzyme-bound NADH fluorescence, which occurred within the mixing time. Because of the higher background due to the reaction, a net enhancement following the rapid quenching could only be observed at the higher serine concentration, where the reaction was being inhibited at a faster rate. (At lower serine concentrations \( F_i > F_{fr} \), whereas at higher serine concentrations \( F_{fr} > F_i \), see "Appendix"). First order rate constants for the enhancement process, determined at 0.1 and 1 mM serine, were found to be the same as when excitation was at 285 nm.

The results presented in this section resolve the discrepancy between the magnitudes of \( k_{obs} \) and \( k_{obs,fr} \). A serine-dependent fluorescence enhancement of enzyme-bound NADH, whose rate correlated with the rate of decay of enzyme activity, was observed during the pre-steady state inhibition of the reaction. Fluorescence quenching by serine of the binary enzyme:pyridine nucleotide complex and fluorescence enhancement by glycine of the enzyme-bound reduced pyridine nucleotide during turnover resulted in the same final equilibrium level of fluorescence. These results are consistent with the quenched state of the binary complex being the state which is directly responsible for the loss of enzyme activity. However, during turnover it is achieved via a different pathway (from a different initial conformation) than in the absence of hydroxypyruvate-P.

**Table 1**

| Serine     | \( k_{obs} \) | \( k_{obs,fr} \) |
|-----------|--------------|-----------------|
| mM        | s⁻¹          | s⁻¹             |
| 0.02      | 3.64         | 2.30            |
| 0.05      | 5.33         | 5.42            |
| 0.1       | 6.55         | 7.92            |
| 1         | 9.35         | 8.22            |

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**Kinetics of Pre-steady State Inhibition Process and Fluorescence Quenching with Glycine – Phosphoglycerate dehydrogenase activity is inhibited by glycine (7). The steady state level of inhibition of hydroxypyruvate-P reduction was determined as a function of glycine concentration and a sigmoid curve was obtained. The Hill numbers were 2.38 and 2.34 and the \( k_{obs} \) values obtained for the corresponding concentrations of the enzyme vary. The invariant \( k_{obs} \) for glycine of 9 s⁻¹ at pH 8.5 did not differ significantly from the 8 s⁻¹ upper limit for \( k_{obs} \) for serine.

At pH 7.5, between 0.75 and 200 mM glycine, \( k_{obs,fr} \) increased from 1 to 1.4 s⁻¹. These values were about 30 to 50% higher than the \( k_{obs,fr} \) values measured for the corresponding (in terms of degree of fluorescence quenching) serine concentrations. This difference was obtained using the same enzyme preparation on the same day.

At pH 8.5, \( k_{obs,fr} \) was invariant at 1.1 s⁻¹ between 0.75 and 200 mM glycine, and then increased between 2 and 200 mM glycine to 2 s⁻¹. This pattern was similar to the pattern seen with serine, except that the amount of the increase was lower with glycine. The \( k_{obs,fr} \) values for the lower glycine concentrations (1.1 s⁻¹) were the same as the \( k_{obs,fr} \) values for the higher serine concentration. The rise in \( k_{obs,fr} \) for glycine was not due to a zwitterion ionic strength effect. The presence of 200 mM D-valine, which did not itself quench fluorescence, did not alter \( k_{obs} \) for 2 mM glycine. It was of interest that 200 mM

![Fig. 9. Dependence of \( k_{obs} \) on glycine concentration. Experiments such as those described in Fig. 2B were performed. A, pH 7.5. Reagent concentrations after mixing: 375 μM hydroxypyruvate-P, 20 μM NADH, and 0.4 μM enzyme. The concentration of glycine was varied. B, pH 8.5. Reagent concentrations after mixing: 375 μM hydroxypyruvate-P and 25 μM NADH; 0.4 ( ), 0.75 ( ), or 1.5 ( ) μM phosphoglycerate dehydrogenase was used. The concentration of glycine was varied. The closed circles represent \( k_{obs} \) as evaluated from semilog plots such as those shown in Fig. 2B, inset; O, the final steady state inhibited level achieved in these experiments. The steady state inhibited rates were obtained from the same traces as the \( k_{obs} \) values and compared to the rate of fully active enzyme, which was determined in a control experiment.](http://www.jbc.org/doi/10.1083/jbc.281.53.1533)
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**FIG. 10. Dependence of \( k_{\text{on,\text{pH}}} \) and the amplitude of the decrease in fluorescence on glycine concentration.** Experimental conditions were identical with those in Fig. 4 except that varying glycine concentrations were used in place of serine. \( k_{\text{on,\text{pH}}} \) (○) was evaluated from semilog plots such as those shown in Fig. 4, inset. The amplitudes (○) of the decrease in fluorescence were evaluated from the same traces as the \( k_{\text{on,\text{pH}}} \) values. The amplitudes cannot be directly compared with the amplitudes shown in Figs. 5 and 6 because the experiments shown in these figures were performed without the photometric log amplifier, with a different xenon lamp, and with an altered optical alignment.

L-valine, which only quenched fluorescence by one-third of maximum, exhibited a \( k_{\text{on,t}} \) of 2 s\(^{-1}\).

**Ultraviolet Difference Spectrum** — The ultraviolet difference spectrum between the enzyme·reduced pyridine nucleotide complex and the enzyme·reduced pyridine nucleotide complex plus serine at pH 7.5 is shown in Fig. 11. Serine causes an increase in absorbance in the region between 265 and 295 nm, with peaks at 272 nm (Δε = 600 M\(^{-1}\) cm\(^{-1}\)), 281 nm (Δε = 1000 M\(^{-1}\) cm\(^{-1}\)), and 288 nm (Δε = 1500 M\(^{-1}\) cm\(^{-1}\)). The difference spectrum at pH 8.5 was qualitatively the same, but the magnitude of the increase in absorbance was about twice as large.

**DISCUSSION**

**Evidence That Serine Inhibits Phosphoglycerate Dehydrogenase via Allosteric Mechanism Involving Reversible Conformational Change** — There are at least three types of mechanisms by which serine might inhibit phosphoglycerate dehydrogenase.

Serine may bind at or near the active site and thereby directly interfere with substrate or coenzyme binding or with amino acid residues involved in catalysis. This mechanism can be ruled out for the following reasons.

1. Serine inhibition of the reaction in either direction was not competitive with respect to either substrate or coenzyme (7, 10).

2. If serine acted by competing for the NADH binding site or a portion of the NADH-binding site, thus preventing NADH binding, saturating amounts of serine, which inhibit phosphoglycerate dehydrogenase by greater than 99%, should completely eliminate both enzyme-bound NADH fluorescence and the quenching of protein fluorescence by enzyme-bound NADH (8). Saturating amounts of serine only quenched enzyme-bound NADH fluorescence by one-third (at pH 8.5) excited either directly or through the protein. This quenching was due to a change in the environment of the bound coenzyme, rather than to the release of some of it into solution, since the protein fluorescence was not concomitantly affected by serine (8).

Even in the presence of serine, the fluorescence of enzyme-bound NADH was still considerably enhanced over that of free NADH in solution. Fluorescence titrations of the enzyme with NADH in the presence and absence of serine yielded identical results with respect to the number of titratable sites and the tightness of binding' (8).

3. There is evidence that hydroxypyruvate-P remains bound to the enzyme in the presence of serine. At pH 8.5, the \( k_{\text{on}} \) for steady state serine inhibition was 5.1 \( \mu \)M (Fig. 1), whereas the serine concentration which resulted in a half-maximal fluorescence quenching of the enzyme·NADH complex by serine was 9.5 \( \mu \)M (Fig. 6). This titration was repeated in a static equilibrium experiment and a value of 8.8 \( \mu \)M was obtained. At pH 8.5, the serine concentration required for the half-maximal increase in \( k_{\text{on,\text{pH}}} \) was 55 \( \mu \)M (Fig. 3), whereas the serine concentration required for the half-maximal increase in \( k_{\text{on,\text{pH}}} \) was 100 \( \mu \)M (Fig. 6). The major form of the enzyme during the steady state reaction is the ternary complex phosphoglycerate dehydrogenase·hydroxypyruvate·NADH (19). These observations show that hydroxypyruvate-P...
phosphate-P influences the interaction of serine with phosphoglycerate dehydrogenase by increasing the apparent affinity of enzyme for serine. This implies that serine does not act by occluding hydroxyphosphate-P from its binding site.

There was no competitiveness between serine and NADH or hydroxyphosphate-P in the pre-steady state inhibition kinetics. This was shown by varying the serine concentration (Fig. 3), the NADH concentration (Fig. 3), and the hydroxyphosphate-P concentration.

A mechanism whereby serine binds to phosphoglycerate dehydrogenase at or near the active site and directly interferes with amino acid residues involved in catalysis would require bimolecular pre-steady state inhibition kinetics. k_{obs} should increase linearly and indefinitely with serine concentration. Fig. 3 shows that at pH 8.5, the k_{obs} versus serine concentration curve was hyperbolic rather than linear, and k_{obs} reached a saturating value of 8 s⁻¹ at higher concentrations. At pH 7.5, k_{obs} increased very slightly with serine concentration. Although the increase appears to be linear on the log scale, it was actually hyperbolic. Also, the rate of increase of k_{obs} with serine concentration was insignificant in terms of the magnitude of the bimolecular association rate constant expected for a ligand which binds at micromolar concentrations.

A bimolecular rate constant in the range of 10⁵ to 10⁶ M⁻¹ s⁻¹ would be expected (16). Thus the binding of 0.5 mM serine is most likely completed within the mixing time in a stopped flow experiment, and the binding of serine cannot be the rate-limiting process for serine inhibition, since the inhibition process was clearly observed on the stopped flow instrument.

One might consider the possibility that serine does inhibit phosphoglycerate dehydrogenase directly by binding at or near the active site, but serine can only bind to certain conformations of the enzyme and not to others. The rate of serine binding and therefore k_{obs} could be limited by the rate of an isomerization involved in the catalytic mechanism. However, experiments in which enzyme, NADH, and serine were mixed with hydroxyphosphate-P (Fig. 2C), demonstrated that the preincubation of serine with the enzyme-NADH complex was sufficient to eliminate the pre-steady state inhibition transient. The pre-steady state inhibition experiments were performed by mixing enzyme and NADH with serine and hydroxyphosphate-P. Since serine can interact upon mixing with the enzyme-NADH complex, the mechanism which is being considered is untenable.

The above considerations demonstrate conclusively that serine inhibits phosphoglycerate dehydrogenase via an indirect, allosteric mechanism.

Several lines of evidence argue against an allosteric mechanism in which serine induces a reversible change in the polymeric state of the enzyme, with either the polymerized or depolymerized form being inactive.

1. Velocity centrifugation experiments have shown that phosphoglycerate dehydrogenase has the same sedimentation coefficient in the presence and absence of serine (9).
2. k_{obs} was independent of phosphoglycerate dehydrogenase concentration. This result excludes a protein polymerization mechanism in which the polymerization process is rate-limiting.
3. The steady state inhibition of phosphoglycerate dehydrogenase as a function of serine concentration was virtually independent of enzyme concentration over a 100-fold range (0.01 to 1 μM), as was the uninhibited steady state turnover number. This argues against the existence of different molecular weight forms of phosphoglycerate dehydrogenase having different kinetic characteristics, since the relative prevalence of such forms should be a function of enzyme concentration.

4. Most enzyme polymerization or depolymerization processes which have been studied occur in the time span of minutes rather than seconds (17).

The third possible mechanism, for which we have accumulated supporting evidence, is that serine inhibits phosphoglycerate dehydrogenase via an allosteric mechanism in which the rapid binding of serine to the enzyme is followed by a slower reversible isomerization of the enzyme. The isomerization results either in the inability of phosphoglycerate dehydrogenase to bind substrate or coenzyme, or a reorientation of the groups at the active site which makes the active site incapable of catalyzing the reaction.

The pre-steady state inhibition kinetics were consistent with serine inhibition occurring via a rapid bimolecular process (serine binding to the enzyme) followed by at least one monomolecular process. The evidence favoring this scheme is the first order nature of the transition of the enzyme reaction from the active to the inhibited rate and the nature of the enzyme and serine concentration dependencies of k_{obs}.

The monomolecular processes could correspond to a conformational change in the enzyme or to the dissociation of hydroxyphosphate-P or NADH from the enzyme following a conformational change.

Evidence has been presented against the proposal that serine acts by directly competing for the binding site or a portion of the binding site of the substrate or coenzyme. Many of the same arguments can be used to show that hydroxyphosphate-P or NADH are not occluded from the enzyme as the result of a serine-induced conformational change. An additional point should be made. The dissociation rate of hydroxyphosphate-P from phosphoglycerate dehydrogenase was 31.5 s⁻¹ at pH 8.5 (see accompanying paper (15)), whereas the maximum value of k_{obs} at high serine concentrations was 8 s⁻¹. Thus the dissociation of hydroxyphosphate-P from the enzyme could not be the rate-limiting step in the pre-steady state inhibition process. One might argue that the rate of dissociation of hydroxyphosphate-P from the serine-induced conformation was slower than from the active conformation. However, if hydroxyphosphate-P is occluded from the inactive conformation, its dissociation rate should be much greater than, not less than, its dissociation rate from the active conformation.

Thus the monomolecular process(es) responsible for serine inhibition must be a conformational change or a series of conformational changes in phosphoglycerate dehydrogenase which result in a greatly decreased (>90%) ability, if not a total inability, of the active site to catalyze the reaction. This corresponds to a V-type allosteric enzyme (2) as previously proposed (7).

No partially active intermediate conformations accumulated during the pre-steady state portion of the inhibition process, as evidenced by the fact that the entire process could be described by a single first order rate constant. However, there may be active intermediates, inactive intermediates, or partially active intermediates which did not accumulate.

In principle, serine could quench the fluorescence of the enzyme-NADH complex directly by binding at or near the active site or indirectly via an allosteric mechanism. The entire quenching process was described by a single first order rate constant (k_{obs}) which had qualitatively the same enzyme and serine concentration dependencies as k_{obs}.

Arguments similar to those made above for serine inhibition indi-
cate that the fluorescence quenching was the result of a conformational change in the enzyme. If the enzyme-bound NADH is viewed as a fluorescent probe at the active site, then it is clear that the organization of the active site was changed by the presence of serine. The homogeneity of the fluorescence quenching process indicates that no intermediate conformations having intermediate fluorescence properties accumulated during that process. However, intermediate conformations which have enzyme-bound reduced pyridine nucleotide fluorescence properties identical with either the initial or the final state cannot be ruled out. Intermediate conformations which have intermediate fluorescence properties but did not accumulate also cannot be ruled out.

Arguments analogous to those made for serine indicate that the inhibition of phosphoglycerate dehydrogenase by glycine and the fluorescence quenching of the enzyme:NADH complex by glycine were also the result of a conformational change in the enzyme.

The ultraviolet difference spectrum between the enzyme:NADH complex and the enzyme:NADH complex plus serine (Fig. 11) shows that the interaction between serine and the enzyme:NADH complex resulted in a change in the environment of some of the aromatic amino acid residues of the enzyme. The change could be due to a modification of the environment of the serine-binding site upon the binding of serine to the enzyme or to a conformational change induced by serine. Preliminary stopped flow experiments have indicated that the rate of increase in absorbance at 287 nm when serine was mixed with the enzyme:NADH complex was the same as the rate of fluorescence quenching of the enzyme:NADH complex by serine. Thus the increase in absorbance at 287 nm was probably the result of the same conformational change which resulted in the fluorescence quenching.

Pathway of Allosteric Transition - Koshland et al. (3) have pointed out that there are two pathways by which an allosteric transition may occur. One pathway requires that a spontaneous equilibrium exist between two conformational states of the protein (or monomers). An effector molecule does not directly induce a conformational transition in the protein molecule to which it binds, but preferential binding of the effector to one of the conformational states displaces the conformational equilibrium in the direction of that state (equilibrium displacement transition). If symmetry is maintained among subunits, this pathway corresponds to the model presented by Monod, Wyman, and Changeux (2). Alternatively, the binding of an effector molecule to a protein molecule can directly induce a conformational transition in that protein molecule (ligand induced transition). The sequential-type allosteric model (3) may proceed via either pathway.

Mechanism 1 (Fig. 12) presents an equilibrium displacement model for the allosteric transition of phosphoglycerate dehydrogenase which is consistent with the serine concentration dependence of $k_{obs}$ at pH 8.5. The R and T forms are in a pre-equilibrium, and serine binds preferentially to T ($K_{ser,T} < K_{ser,R}$). There are two pathways by which T-Ser can be reached when serine is mixed with R. At lower serine concentrations, serine binds exclusively to T and displaces the R:T equilibrium, causing an allosteric transition from R to T (pathway 1). At higher serine concentrations, serine binds to R and destabilizes it, so that the R-Ser to T-Ser transition occurs at a faster rate than the R to T transition. Thus, at lower serine concentrations the allosteric transition takes place exclusively via pathway 1. As the serine concentration increases and binding to R begins to occur, there is a competition between the two pathways, and the predominant pathway is converted to pathway 2 as R becomes saturated with serine.

The basis for the model is the difference between $k_{obs}$ for the steady state inhibition by serine (5.1 μM) and the serine concentration which resulted in a half-maximal increase in $k_{obs}$ (55 μM). The increase in $k_{obs}$ began to occur at 20 μM serine, a concentration at which the steady state level of inhibition was already 94% (Fig. 3). Thus a process was beginning to occur at a serine concentration which was already capable of bringing about the allosteric transition. That process, according to Mechanism 1, was the binding of serine to R. This model is essentially equivalent to the Monod, Wyman, Changeux model with nonexclusive effector binding.

A ligand-induced mechanism is also consistent with the serine data (Mechanism 2). One can postulate the existence of two classes of serine binding sites, a tight (class A) and a loose (class B) class. At low serine concentrations, serine binds to the tight sites only and induces the allosteric transition. As the serine concentration is raised, binding to the loose sites begins to occur, as well. Enzyme molecules which have serine bound to both the tight and loose sites isomerize at a faster rate than those which have the tight sites occupied. There is no teleological reason for there being a class of loose serine binding sites, since binding to the tight sites would be sufficient to cause virtually complete inhibition of the enzyme. It is possible, that the class B sites are the "glycine" binding sites, and at present there is no evidence as to whether serine and glycine bind to the enzyme at the same or different sites. Another possibility is negative cooperativity in serine binding. The steady state velocity of the catalytic reaction in the direction of phosphoglycerate oxidation as a function of 3-phosphoglycerate concentration yielded a biphase double reciprocal plot (10, 15), suggesting negative cooperativity in 3-phosphoglycerate binding. In addition, the binding of NADH to the enzyme exhibits negative cooperative behavior (8).

The pH 7.5 data are consistent with Mechanism 1, with the following alternative possibilities: the R-Ser to T-Ser isomerization occurs at about the same rate as the R to T isomerization; or serine does not bind to R at all. The data are also consistent with Mechanism 2 in which binding of serine to the loose sites does not affect the rate of the allosteric transition, or in which serine does not bind to the loose sites at this pH.

The qualitative patterns of the serine concentration dependence of $k_{obs}$ were the same as those of $k_{obs}$. Using the same reasoning applied above, it is apparent that both Mechanisms 1 and 2 can explain the allosteric transition of the enzyme:NADH complex.

The glycine concentration dependence of $k_{obs}$ and $k_{obs}$.
was also examined (Figs. 9 and 10), and provides evidence favoring the ligand-induced pathway. The pH 7.5 data was essentially identical with the serine data, except that \( k_{obs,1/2} \) might be about 30% higher for glycine than for serine. If this difference is real, it provides evidence that the ligand-induced pathway is operating at pH 7.5. This follows from the assumption that in an equilibrium displacement mechanism, at low glycine concentrations, only binding to T would occur \( \left( K_{D,T} \ll K_{D,n,T} \right) \). (If this were not the case, limits would be introduced on the extent to which the R-T equilibrium could be shifted by glycine (10), and saturating the enzyme with glycine would not result in complete inhibition of enzyme activity. In fact, greater than 90% inhibition of enzyme activity is achieved at high glycine concentrations.) The R to T transition, which would be the rate-limiting step at low ligand concentrations, does not involve ligand, so its rate would be independent of the nature of the ligand which is binding to T to displace the equilibrium. Thus at low ligand concentrations, one would expect the nature of the ligand to influence the rate of an allosteric transition in an equilibrium displacement mechanism. However, it is reasonable that a ligand-induced R-Gly to T-Gly transition would occur at a different rate than a ligand-induced R-Ser to T-Ser transition.

The patterns of the pH 8.5 data obtained with serine and glycine differed. \( k_{obs,1} \) for glycine was independent of glycine concentration, and its value did not differ significantly from the saturation value of \( k_{obs} \) at the higher serine concentrations. These results are inconsistent with the equilibrium displacement mechanism because at low ligand concentrations the magnitude of \( k_{obs} \) was much greater for glycine than for serine. However, the results are consistent with Mechanism 2 in which glycine binds to the class B sites and induces the allosteric transition at the same rate as when serine binds to these sites.

Similarly, the difference between the values of \( k_{obs,1/2} \) at low glycine compared with low serine concentrations at pH 8.5 is inconsistent with Mechanism 1. In terms of Mechanism 2, the correspondence of the values of \( k_{obs,1} \) at low glycine and high serine concentrations suggests that at low glycine concentrations glycine binds to the class B sites and induces the allosteric transition at the same rate as when serine binds to these sites. At higher glycine concentrations, glycine binds to the class A sites of the enzyme-NADH complex as well. The rate of the allosteric transition when glycine is occupying both sites could be greater than the rate when glycine is only occupying the class B sites.

The difference in the qualitative pattern between the dependence of \( k_{obs,1/2} \) and \( k_{obs,1} \) on glycine concentration at pH 8.5 is most likely due to differences between the enzyme when it is involved in catalysis and the enzyme-NADH complex.

Two alternative models for the pathway of the allosteric transition have been presented. Although both of these mechanisms are intuitively appealing, they are only qualitative first approximations. They do not take into account the subunit nature of the enzyme. The apparent interactions which occur among the subunits, or the possibility of there being kinetic intermediate conformations along the pathway of the allosteric transition. The models can be viewed as a framework for planning future work. At this point, the data favors Mechanism 2. Equilibrium binding experiments with radioactive serine, along with binding competition experiments between radioactive serine and cold glycine should help to clarify the allosteric mechanism. Temperature jump relaxation experiments could begin to define the intermediate processes which may be involved. Janin and Iwatsubo (14) detected a kinetic intermediate in the allosteric transition of homoserine dehydrogenase using this technique.

**Conformational Transition Which Is Responsible for Inhibition of Phosphoglycerate Dehydrogenase by Serine**—In the accompanying paper (15), we proposed the following minimal reaction scheme for the ternary complex interconversion of phosphoglycerate dehydrogenase:

\[
E\cdot NADH\cdot hydroxy	pyruvate-P (1) \xrightarrow{\Delta} E\cdot NADH\cdot hydroxy	pyruvate-P + H^+ (2) \xrightarrow{\Delta} E\cdot NAD^+\cdot 3\cdot p (3) \xrightarrow{\Delta} E\cdot NAD^+\cdot 3\cdot p\cdot phosphate (4)
\]

Steps 1, 2, and 4 are isomerizations. Step 3 is the hydride transfer. At pH 7.5 and 8.5, isomerization 2 was shown to be the rate-determining step in the overall steady state reaction, and isomerization 1 was shown to be relatively rapid. Isomerization 1 corresponds to the 50-ms lag observed in Fig. 8A.

This understanding of the catalytic mechanism of phosphoglycerate dehydrogenase provides further insight into the nature of the change in enzyme structure responsible for the inhibition of phosphoglycerate dehydrogenase by serine. Table 1 shows that the rate of fluorescence enhancement caused by serine during the pre-steady state portion of the inhibition process at pH 8.5 corresponded to the rate of the transition of the enzyme-NADH-hydroxy	pyruvate-P complex (which is the predominant form of the enzyme after mixing) to the inactive form of the enzyme. The lag in the enhancement process indicates that the transition of the \( E\cdot NAD\cdot hydroxy	pyruvate-P \) complex is similar to or identical with \( E\cdot NAD\cdot hydroxy	pyruvate-P + H^+ \) to the inactive form of the enzyme. (Conversely, the lag in the enhancement process is additional evidence for the existence of isomerization 1.)

The rapid quenching of the enzyme-NADH complex fluorescence by hydroxy	pyruvate-P explains why a fluorescence enhancement is induced by serine in the presence of hydroxy	pyruvate-P, whereas a fluorescence quenching is induced in the absence of hydroxy	pyruvate-P. The same equilibrium level of fluorescence of the inactive complex is achieved, but from different starting points. The existence of isomerization 1 explains why the magnitudes of \( k_{obs,1/2} \) and \( k_{obs,1} \) differ. \( k_{obs,1/2} \) represents the rate of the allosteric transition of the binary enzyme-NADH complex to the inactive form of the enzyme, and \( k_{obs,1} \) represents the rate of the allosteric transition of \( E\cdot NAD\cdot hydroxy	pyruvate-P \) to the inactive form of the enzyme. Thus the conformation of the enzyme-NADH complex is similar to or identical with \( E\cdot NAD\cdot hydroxy	pyruvate-P \) which also undergoes the allosteric transition at a slower rate than \( E\cdot NAD\cdot hydroxy	pyruvate-P \).

Correlations between the rate of an inhibition process and the rate of a structural change have been made in other systems. Janin and Iwatsubo (14) correlated the rates of several spectroscopic changes with the rate of inhibition of homoserine dehydrogenase by threonine as first measured by Barber and Bright (13). Gutfreund et al. (19) correlated the rate of inhibition of lactate dehydrogenase by pyruvate with the rate of formation of the enzyme-NAD\cdot pyruvate complex.

\( k_{obs,1} \) was independent of hydroxy	pyruvate-P concentration, even at concentrations of hydroxy	pyruvate-P below the \( K_M \). This provides evidence that subunits act independently. When
fewer than half of the subunits are in the process of catalyzing the reaction, and are in conformation $E_{c}$:NADH-hydroxy-
pyruvate-P, the rate of their transition to the inactive conformation is not influenced by the subunits which are not catalyzing the reaction.

**Acknowledgments** – We are grateful to Dr. Harold Bright for the use of his stopped flow instrument and for helpful discussions. We would also like to thank Carl Fuller for his help in performing the difference spectra.

**APPENDIX**

The traces shown in Fig. 8, A and B, were obtained using the same time and millivolt scales. The observed fluorescence enhancement is actually the sum of the fluorescence increase of the phosphoglycerate dehydrogenase-bound NADH and the fluorescence decrease due to the oxidation of NADH in the reaction, which is being shut off at an exponential rate due to the serine inhibition. Thus assuming that the fluorescence enhancement of the enzyme-bound NADH is a first order process,

$$F(t) = F_{0}(1 - e^{-k_{obs}t}) - F_{i}(1 - e^{-k_{obs}t})$$

$F(t)$ is the observed fluorescence (above the rapidly quenched level) at time $t$ after mixing; $F_{0}$ is the total magnitude of the increase in enzyme-bound NADH fluorescence caused by serine; $F_{i}$ is the magnitude of the fluorescence decrease, due to the reaction, produced by inhibitable enzyme during the pre-steady state inhibition process, and $k_{obs}$ and $k_{obs}$ are the respective first order rate constants. The assumption is also being made that the reaction is being completely shut off, which is the case for the serine concentrations used in these experiments.

If $k_{obs} = k_{obs}$, then $F(t) = (F_{0} + F_{i})(1 - e^{-k_{obs}t})$. Thus the kinetics of the observed enhancement process would be described by a single first order rate constant equal to $k_{obs}$. However, if $k_{obs}$ differed greatly from $k_{obs}$, the observed kinetics of enhancement would be the sum of two exponentials, and could not be described by a single observed first order rate constant. (At the higher serine concentrations at which the enzyme activity is being turned off at a greater rate, $F_{obs} = F_{i}$, so that deviations from a single exponential process would be minimal, even if $k_{obs}$ differed greatly from $k_{obs}$). However, in this case the observed kinetics of enhancement are a close approximation to the actual kinetics of enhancement of enzyme-bound NADH fluorescence.)

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J. Biol. Chem. 1977, 252:1527-1538.

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