The inhibition of *Escherichia coli* d-3-phosphoglycerate dehydrogenase by L-serine is positively cooperative with a Hill coefficient of ~2, whereas the binding of the inhibitor, L-serine, to the apoenzyme displays positive cooperativity in the binding of the first two serine molecules and negative cooperativity in the binding of the last two serine molecules. An earlier report demonstrated that the presence of phosphate appeared to lessen the degree of both the positive and negative cooperativity, but the cause of this effect was unknown. This study demonstrates that the presence of intrinsically bound NADH was responsible to a substantial degree for this effect. In addition, this study also provides evidence for negative cooperativity in NADH binding and for at least two NADH-induced conformational forms of the enzyme that bind the inhibitor in the physiological range. Successive binding of NADH to the enzyme resulted in an increase in the affinity for the first inhibitor ligand bound and a lessening of both the positive and negative cooperativity of inhibitor binding as compared with that seen in the absence of NADH. This effect was specific for NADH and was not observed in the presence of NAD⁺ or the substrate α-ketoglutarate. Conversely, the binding of L-serine did not have a significant effect on the stoichiometry of NADH binding, consistent with it being a V-type allosteric system. Thus, cofactor-related conditions were found in equilibrium binding experiments that significantly altered the cooperativity of inhibitor binding. Since the result of inhibitor binding is a reduction in the catalytic activity, the binding of inhibitor to these NADH-induced conformers must also induce additional conformations that lead to differential inhibition of catalytic activity.

The binding of L-serine to PGDH has been studied and shown to exhibit characteristics of both positive and negative cooperativity (5). L-Serine binds to PGDH at the interface between two adjacent regulatory domains with two molecules of L-serine binding at each of the two regulatory domain interfaces (4). Each serine molecule forms hydrogen bonds with adjacent domains forming a hydrogen bond network across the non-covalent interface. This network appears to tether the domains together and results in inhibition of catalytic activity (4, 6). It has been proposed that the potential binding of two effector molecules at a single interface could explain the negative cooperativity of ligand binding if a single effector molecule was capable of stabilizing the association of the two domains (5) to the extent that it could exclude, or partially exclude, the binding of the second ligand.

L-serine binding was shown to display significant differences in binding behavior in the presence of phosphate (7). This manifested itself in an alteration of the degree of both the positive and negative cooperativity of serine binding as well as the relative sensitivity of PGDH to serine concentration. This effect seemed to be unique to phosphate among the buffers tested in that several other buffers acted similarly to each other but differently from phosphate. The nature of this "phosphate effect" was unknown at the time, but it was proposed that the phosphate ion might interact at the active site since phosphate groups are part of the natural substrates, 3-phosphoglycerate and NAD⁺ (7). It was suggested that linkage between the active sites and the serine binding sites could result in a more open form of the regulatory interface in response to phosphate interaction at the active site, which might then manifest itself in a greater ease of serine binding and a lessening of cooperative effects.

If this phosphate effect is the result of interaction at the active site, it suggests that the natural substrates of the enzyme, which contain phosphate groups, would also have a measurable effect on the serine binding characteristics. Such reciprocal effects, that is, the binding of ligand at one site affecting the binding of another ligand at a separate site, is not uncommon (8). In fact, this is essentially the basis for K-type regulation in allosteric systems (8).

PGDH has been reported to be basically a V-type regulatory system (2). In other words, binding of effector has its major effect on the velocity of the enzyme rather than on substrate binding. In this respect and in light of recent observations (7), it was of interest to explore the potential significance of this further.

Sugimoto and Pizer (1) reported that purified PGDH contained approximately two molecules of intrinsically bound NADH. The amount of NADH bound could be observed by fluorescence resonance energy transfer between tryptophan residues in the enzyme and the bound NADH (1). Sugimoto and Pizer (1) also reported that the fluorescence signal due to bound d-3-Phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95) from *Escherichia coli* catalyzes the first committed step in the phosphorylated pathway of l-serine biosynthesis. In turn, l-serine inhibits PGDH catalytic activity in an allosteric, cooperative manner (1–3). PGDH is a homo-tetrameric enzyme that contains four active sites and four l-serine binding sites (4).

The abbreviations used are: PGDH, d-3-phosphoglycerate dehydrogenase; DTT, dithiothreitol.

**Cofactor Binding to *Escherichia coli* d-3-Phosphoglycerate Dehydrogenase Induces Multiple Conformations Which Alter Effector Binding**

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*The abbreviations used are: PGDH, d-3-phosphoglycerate dehydrogenase; DTT, dithiothreitol.
NADH was affected by the presence of l-serine, but they concluded that serine only altered the fluorescence properties of the enzyme rather than the amount of bound NADH.

To further explore the modulation of cooperativity in PGDH, the effect of cofactors and substrates on the l-serine binding characteristics of PGDH were investigated. This report provides evidence that although some effect of phosphate buffer is seen, a major effect on serine binding is the direct influence of the binding of NADH but not of NAD or α-ketoglutarate.

MATERIALS AND METHODS

PGDH was produced and isolated as described previously (9, 10). All experiments were performed with PGDH from C. freundii, which is a form of the enzyme in which the 4 cysteine residues in each subunit are converted to alanine residues (11). After cell lysis and ammonium sulfate fractionation, the enzyme was purified by affinity chromatography on a column of 5′-AMP-Sepharose and eluted with NADH. After elution, the enzyme was dialyzed directly against either 20 mM potassium phosphate buffer, pH 7.5, 1 mM DTT, 1 mM EDTA or 20 mM Tris buffer, pH 7.5, 1 mM DTT, 1 mM EDTA. In some instances, as described below, the NADH was converted to NAD⁺ by the addition of α-ketoglutarate prior to dialysis. This conversion was monitored by following the fluorescence of the solution at 420 nm as described below. Catalytic activity was monitored by the change in absorbance at 340 nm due to the conversion of NADH to NAD⁺ at pH 7 (12) using α-ketoglutarate as the substrate (13). Protein concentration was determined by quantitative amino acid analysis.

Serine binding was measured by equilibrium dialysis in 200-μl dialysis chambers (Sialomed, Inc., Columbia, MD) purchased from the Nest Group (Southborough, MA). Dialysis was performed for 16 h with [3H]l-serine in appropriate concentrations of unlabeled l-serine. Cells were sampled in triplicate, and the average of 10-min counts was used to calculate concentrations of free and bound l-serine. The nominal PGDH concentration was 5–10 μM tetramer in all binding experiments. When indicated, NADH, NAD⁺, and α-ketoglutarate were present in solution at 100 μM, 100 μM, and 5 mM, respectively. The dissociation constants for NADH and NAD⁺ were 0.05 and 8 μM, respectively (1), and the K₅₅ for α-ketoglutarate is 0.3 mM. Serine binding data were fit to the Adair equation for four binding sites using Kaleidograph (Synergy Software) as described previously (5, 14).

Fluorescence experiments were performed on a PerkinElmer Life Sciences LS-50B luminescence spectrometer. NADH binding was determined under conditions for stoichiometric binding, which is defined as a concentration of the acceptor that is at least 10 times greater than the dissociation constant of the ligand (15). Excitation was performed at 295 nm, and emission was monitored at 420 nm (1). Fluorescence intensity was corrected for dilution as NADH binds to protein, the Fₐ/F₀ rises in response to the resonance energy transfer. After the enzyme is saturated with ligand, the remaining increase in Fₐ/F₀ is due to free NADH in solution. Extrapolation of the slopes of these two responses gives the moles of NADH bound per moles of tetrameric PGDH at their point of intersection (15). When indicated, l-serine was present at a concentration of 200 μM. Fluorescence scans were performed with excitation at 295 nm and emission monitored from 300 to 450 nm. NADH concentration was determined by absorbance at 340 nm using a molar extinction coefficient of 6.22 × 10⁵ M⁻¹ cm⁻¹ (16).

RESULTS

Fig. 1 shows the fluorescence spectrum of PGDH after isolation from a 5′-AMP-Sepharose affinity column followed by dialysis to remove free NADH. The fluorescence at 420 nm is an indication of the amount of NADH bound to the enzyme. Also shown is the spectrum of PGDH after it has been treated with the substrate α-ketoglutarate. Titration of the sample containing intrinsically bound NADH with additional NADH (Fig. 2) produces an increase in the fluorescence at 420 nm, which corresponds to the binding of ~2 additional NADH per tetramer, which is consistent with there being ~2 mol of NADH bound/tetramer in the isolated enzyme.

Treatment of PGDH containing intrinsically bound NADH with α-ketoglutarate results in a decrease of the signal at 420 nm due to the conversion of bound NADH to NAD⁺ (Fig. 1). After dialysis, samples titrated with NADH (Figs. 3 and 4) show that they are capable of binding ~4 NADH/tetramer.

Thus, the NAD⁺ produced by reaction with α-ketoglutarate either dissociates from the enzyme during dialysis or can be freely displaced by NADH if it is still bound to the enzyme.

It was reported previously that binding of l-serine to PGDH in phosphate buffer appeared to exhibit a lesser degree of cooperativity than binding in Tris buffer (5, 7). However, this was done without prior removal of intrinsically bound NADH. To explore this further, serine binding was performed with enzyme that was depleted of NADH and dialyzed with phosphate buffer. The binding curves measured in the absence and presence of saturating levels of NADH, NAD⁺, and α-ketoglutarate are shown in Fig. 5. The binding parameters derived from fitting the data to the Adair equation are shown in Table I. Serine binding to PGDH in the presence of saturating NADH reduces both the positive and negative cooperativity of binding as compared with binding in phosphate in the absence of NADH or in the presence of NAD⁺ or α-ketoglutarate.
binding in phosphate in the presence of NADH from this study is similar to the binding in phosphate reported previously (7) in that the degree of apparent positive cooperativity is decreased and the binding of the fourth ligand is now measurable. The effect of NADH on serine binding in Tris buffer shows similar behavior in the lessening of positive cooperativity but not the negative cooperativity (Fig. 6).

Differential effects of NADH levels on the serine binding characteristics of PGDH can also be observed. The Scatchard analyses in Fig. 7 are derived under conditions in which NADH is depleted from the enzyme, when only intrinsically bound NADH remains on the enzyme (after dialysis), of when the enzyme is saturated with NADH. The decrease in the concavity of the Scatchard plots shows that the degree of cooperativity observed progressively decreases as the NADH status progresses from complete absence to saturation. As expected, the derived dissociation constants listed in Table I show a distinct progression from no NADH to saturating NADH.

The distribution of each effector-bound species, calculated from the Adair constants presented in Table I for PGDH in the absence and presence of saturating NADH in phosphate buffer, is shown in Fig. 8. The nearly 6-fold increase in affinity for the first inhibitor ligand in the presence of NADH significantly enhances the inhibitor occupancy of PGDH at very low serine concentrations. For instance, at 1 mM free L-serine, the percentage of enzyme with at least one inhibitor ligand bound increases from 16 to 50% in the presence of NADH. In addition, the species with at least three inhibitor ligands bound is also significantly enhanced in the presence of NADH. At 5 mM L-serine, this occupancy increases from about 5 to 27%, and at 10 mM L-serine, it increases from 12 to 55%. The population of species with at least two ligands bound is also increased in the presence of NADH but not nearly to the extent as the others.

Because of the effect that the presence of NADH has on serine binding, the effect of serine on NADH binding was also investigated. NADH titrations of NADH-depleted PGDH in the presence of saturating levels of L-serine (Figs. 3 and 4) demonstrate that serine has no appreciable effect on the level of NADH binding in either Tris or phosphate buffer. Serine did appear to

FIG. 3. NADH titration of PGDH in the presence or absence of L-serine in phosphate buffer. Immediately after purification, PGDH was treated with α-ketoglutarate to convert NADH to NAD⁺ and then dialyzed against 20 mM potassium phosphate buffer, pH 7.5, 1 mM DTT, 1 mM EDTA for 18 h. Titrations were performed without added L-serine (●) or in the presence of 200 mM L-serine (■). The y axis is expressed as the fluorescence at 420 nm minus the initial fluorescence before addition of NADH, F-F₀. The x axis is expressed as moles of NADH per moles of protein (tetramer). Solid lines are extrapolations of the initial and final slopes, and dashed lines are extrapolations of the slope intersections to the x axis.

FIG. 4. NADH titration of PGDH in the presence or absence of L-serine in Tris buffer. Immediately after purification, PGDH was treated with α-ketoglutarate to convert NADH to NAD⁺ and then dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA for 18 h. Titrations were performed without added L-serine (●) or in the presence of 200 mM L-serine (■). The y axis is expressed as the fluorescence at 420 nm minus the initial fluorescence before addition of NADH, F-F₀. The x axis is expressed as moles of NADH per moles of protein (tetramer). Solid lines are extrapolations of the initial and final slopes, and dashed lines are extrapolations of the slope intersections to the x axis.

FIG. 5. Binding of L-serine to PGDH in phosphate buffer. Binding of L-serine is expressed as the fractional occupancy (Y) versus the micromolar concentration of free L-serine. Data are expressed as symbols, and the solid lines are the fit of the Adair equation for four sites to the data. Immediately after purification, PGDH was treated with α-ketoglutarate to convert NADH to NAD⁺ and then dialyzed against 20 mM potassium phosphate buffer, pH 7.5, 1 mM DTT, 1 mM EDTA for 18 h. L-Serine binding to PGDH in the presence of 100 mM NADH (●), 100 mM NAD⁺ (■), 5 mM α-ketoglutarate (○), or no added ligand (▼).
Binding of L-serine is expressed as the fractional occupancy (μM DTT, 1 mM EDTA or 20 mM Tris buffer, pH 7.5, 1 mM DTT, 1 mM EDTA) for four sites to the data. Immediately after purification, PGDH was treated with phosphate ion as well as bound NADH. Data are expressed as dissociation constants (μM) and intrinsic site dissociation constants as Ki.

Cofactor Alters Inhibitor Binding in PGDH

We reported previously (7) that phosphate buffer appeared to enhance the fluorescence slightly in phosphate buffer (Fig. 3), but the number of NADH molecules bound was not altered.

**DISCUSSION**

We reported previously (7) that phosphate buffer appeared to have a specific effect on the binding of serine to PGDH that was not evident with other buffers such as Tris, imidazole, and borate. The results of the studies reported here show that the presence of bound NADH also exhibits a significant effect on the binding of L-serine. Furthermore, this effect is specific for the reduced cofactor since it is not observed with NAD +.

The data in Table I, which were derived from the serine binding curves, show that phosphate buffer alone (phosphate – NADH) may slightly reduce the level of positive cooperativity in the binding of the first two ligands as compared with Tris buffer. However, phosphate alone does not appear to alter the negative cooperativity in the binding of subsequent serine ligands. Serine binding in phosphate buffer in the presence of either NAD + or α-ketoglutarate produces very similar binding parameters to that of phosphate alone (phosphate – NADH). It is not until NADH is introduced into the solution that a significant increase in the affinity of the first ligand and a decrease in the negative cooperativity are seen. In regard to the latter, the presence of NADH now brings binding of the last ligand to the negative cooperativity are seen. In regard to the latter, the presence of NADH now brings binding of the last ligand into the measurable range, whereas under all other conditions, binding is too weak to measure. Note that this does not happen in Tris buffer in the presence of NADH. This is consistent with the previous report (7) since no effort was made to remove the bound NADH from the protein used in those studies. Therefore, these results indicate that the phosphate effect reported previously (7) appears to be due to a combination of effects from the phosphate ion as well as bound NADH.

**Table I**

**L-serine binding parameters**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>K1 (μM)</th>
<th>K2 (μM)</th>
<th>K3 (μM)</th>
<th>K4 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris + NADH</td>
<td>2.7 ± 0.4</td>
<td>11.0 ± 0.0006</td>
<td>1.2 ± 0.3</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Tris – NADH</td>
<td>12.1 ± 4.6</td>
<td>2.4 ± 0.003</td>
<td>7.1 ± 2.7</td>
<td>5.2 ± 2.2</td>
</tr>
<tr>
<td>Phosphate + sat NADH</td>
<td>2.5 ± 0.8</td>
<td>92.9 ± 0.0002</td>
<td>5.0 ± 1.6</td>
<td>7.2 ± 2.4</td>
</tr>
<tr>
<td>Phosphate + int NADH</td>
<td>145 ± 25</td>
<td>1380 ± 1770</td>
<td>VL</td>
<td>VL</td>
</tr>
<tr>
<td>Phosphate – NADH</td>
<td>7.0 ± 0.002</td>
<td>7.8 ± 0.001</td>
<td>8.0 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Phosphate + NAD</td>
<td>8.0 ± 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate + α-KG</td>
<td>8.0 ± 0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Binding performed in the presence of saturating NADH.
Binding performed with intrinsically bound NADH.
VL, very large, i.e. > 10^4.
Fitting statistics, X^2, accumulated deviation between data and calculated curve. R, correlation coefficient.
Unreliable due to the large fitting error.*

**Fig. 6.** Binding of L-serine to PGDH in Tris and phosphate buffer. Binding of L-serine is expressed as the fractional occupancy (Y) versus the micromolar concentration of free L-serine. Data are expressed as symbols, and the solid lines are the fit of the Adair equation for four sites to the data. Immediately after purification, PGDH was treated with α-ketoglutarate to convert NADH to NAD + and then dialyzed against either 20 mM potassium phosphate buffer, pH 7.5, 1 mM DTT, 1 mM EDTA or 20 mM Tris buffer, pH 7.5, 1 mM DTT, 1 mM EDTA for 18 h. L-serine binding to PGDH in phosphate buffer with no added ligand (●) or in the presence of 100 μM NADH (square) is shown. 

In the binding of the first two ligands as compared with Tris buffer. However, phosphate alone does not appear to alter the negative cooperativity in the binding of subsequent serine ligands. Serine binding in phosphate buffer in the presence of either NAD + or α-ketoglutarate produces very similar binding parameters to that of phosphate alone (phosphate – NADH). It is not until NADH is introduced into the solution that a significant increase in the affinity of the first ligand and a decrease in the negative cooperativity are seen. In regard to the latter, the presence of NADH now brings binding of the last ligand into the measurable range, whereas under all other conditions, binding is too weak to measure. Note that this does not happen in Tris buffer in the presence of NADH. This is consistent with the previous report (7) since no effort was made to remove the bound NADH from the protein used in those studies. Therefore, these results indicate that the phosphate effect reported previously (7) appears to be due to a combination of effects from the phosphate ion as well as bound NADH.

**Fig. 7.** Scatchard plots of serine binding with varying levels of NADH. Data are plotted as moles of serine bound per moles of enzyme (r) versus moles of serine bound over free serine concentration (r/F). Binding data are presented for PGDH after depletion of bound NADH with α-ketoglutarate (●), with intrinsic NADH present, i.e. after dialysis but without prior depletion of NADH (square), and in the presence of saturating NADH in solution (●).

In the binding of the first two ligands as compared with Tris buffer. However, phosphate alone does not appear to alter the negative cooperativity in the binding of subsequent serine ligands. Serine binding in phosphate buffer in the presence of either NAD + or α-ketoglutarate produces very similar binding parameters to that of phosphate alone (phosphate – NADH). It is not until NADH is introduced into the solution that a significant increase in the affinity of the first ligand and a decrease in the negative cooperativity are seen. In regard to the latter, the presence of NADH now brings binding of the last ligand into the measurable range, whereas under all other conditions, binding is too weak to measure. Note that this does not happen in Tris buffer in the presence of NADH. This is consistent with the previous report (7) since no effort was made to remove the bound NADH from the protein used in those studies. Therefore, these results indicate that the phosphate effect reported previously (7) appears to be due to a combination of effects from the phosphate ion as well as bound NADH.
On the other hand, the ability of PGDH to bind NADH does not appear to be affected by the level of L-serine. This is consistent with the early work of Sugimoto and Pizer (1), who concluded that serine altered the fluorescent properties of the enzyme rather than the amount of NADH bound.

It is difficult to assess the physiologic significance of the effect of NADH since comparable binding parameters are not obtainable when the enzyme is actively turning over substrate and cofactor. It is well known, however, that serine inhibits product formation and cofactor. It is well known, however, that serine inhibits product formation and the reaction products by the associated metabolism, thus drawing the reaction in the forward direction. This in itself would represent a level of control over the production of L-serine or the intermediate products of the pathway. That serine binding appears to be more positively cooperative in the physiological direction in which the enzyme binds NAD$^+$ as a substrate suggests an additional potential for fine-tuning in the level of control. However, Dubrow and Pizer (17) have presented pre-steady state kinetic evidence that suggested that PGDH is not in the same conformation in the enzyme-reduced cofactor complex as it is when it is turning over.

Although these data show a change in effector binding characteristics in response to cofactor, it is difficult to explain the inhibition characteristics in terms of the effector binding pattern alone. Moreover, additional changes in effector binding may occur when both substrate and cofactor are interacting with the enzyme. Thus, care must be taken when trying to interpret the results of individual ligand binding experiments in the context of an enzyme that is actively turning over, especially when conclusions regarding comparative stoichiometry are made.

PGDH has been classified as a V-type enzyme (2), meaning that regulation of activity is basically through modulation of the catalytic turnover rate rather than substrate binding, which is the case for K-type enzymes. The present studies are consistent with this conclusion, but somewhat unexpected is the significant effect of cofactor binding on effector binding without a similar effect in the opposite direction. The differential binding of NADH visualized in Fig. 7, as well as in Figs. 2 and 3, provides evidence for at least two additional conformational states of the protein. These two additional states correspond to enzyme with intrinsically bound NADH and enzyme under conditions of saturating NADH. This observation has at least two implications. First, the enzyme displays negative cooperativity in the binding of NADH. Two sites in the enzyme bind NADH very tightly since enzyme is purified with two NADH molecules bound/tetramer, which do not dissociate during dialysis. Second, in the presence of additional NADH, the last two sites bind NADH with weaker affinity. This observation is not unprecedented for dehydrogenases which bind NADH as a cofactor. For instance, glyceraldehyde-3 phosphate dehydrogenase from rabbit muscle (18) displays increasing dissociation constants for the four sites that range from around $10^{-11}$ for the first site to $10^{-9}$ for the last site.

Consistent with the induced fit theory of Koshland et al. (19), this study presents evidence for at least three different states that bind inhibitor with affinities in the physiological range that clearly differ in their individual site affinity for the inhibitor. One state exists in the absence of NADH binding, and the latter two are induced by NADH binding. The two induced states manifest themselves most strikingly in an increase in affinity for the first ligand to bind and an increase in the overall degree of ligand binding. In addition, there is a progressive loss of cooperative behavior of effector binding as cofactor occupancy increases. Thus, in addition to the negative cooperativity that cannot be accommodated by the Monod model (8), these data are also more consistent with some variation of the Koshland model, which states that the conformation of each subunit changes in turn as it binds ligand. Furthermore, in addition to the multiple states induced by NADH binding, the subsequent binding to these states by the inhibitor, L-serine, must also induce at least one, and probably more, conformational states that lead to inhibition of catalytic activity.

![Fig. 8. Distribution of effector-bound PGDH species. The plots show the calculated fractional distribution of species with one to four bound L-serine molecules as a function of L-serine concentration in phosphate buffer in the absence (top) and presence (bottom) of NADH. Plots for 1 (■), 2 (●), 3 (●), and 4 (▲) L-serines per tetramer are shown.](http://www.jbc.org/)

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Previously, we have shown that mutations in hinge areas between the inhibitor binding domain and the active site can actually uncouple the cooperativity of inhibition from the cooperativity of serine binding (14, 20, 21). If these mutations literally interrupt the flow of conformational information between these two distant sites because of mutations that hinder movement around these hinges, then one might expect them to also interrupt the effect of NADH on inhibitor binding. However, if the mechanisms for these two phenomena are not the same, then a different outcome would be expected. Investigations are now ongoing to test this hypothesis. In either case, these investigations will provide additional insight into the structural basis for the cooperative allosteric regulation of this enzyme.

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Cofactor Binding to *Escherichia coli* d-3-Phosphoglycerate Dehydrogenase Induces Multiple Conformations Which Alter Effector Binding

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