Probing the Regulatory Domain Interface of D-3-Phosphoglycerate Dehydrogenase with Engineered Tryptophan Residues

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D-3-Phosphoglycerate dehydrogenase from Escherichia coli is a homotetrameric enzyme which is allosterically regulated by the end product of its pathway, L-serine. The enzyme binds 4 L-serine molecules at two interfaces formed by the noncovalent association of the regulatory domains. The two domains that comprise each interface are related by an approximately 180° axis of symmetry, and two serine molecules bind at each interface by forming a hydrogen bond network between the domains. A model has been proposed that suggests that serine functions by drawing adjacent domains together and that this in turn translates a conformational change to the active site. A tryptophan residue has been engineered into the helices flanking the regulatory interfaces that displays significant quenching in response to serine binding. Residues on the adjacent subunit appear to be primarily responsible for the tryptophan quenching and thus support the hypothesis that serine binding leads to an increase in the proximity between residues on neighboring subunits. Serine binding studies show that this quenching, as well as inhibition of enzymatic activity, are essentially complete when only two of the four serine binding sites are occupied. The requirement for only one serine per interface is consistent with the notion that the interface is formed by relatively rigid domains and that hydrogen bonding at only a single site is all that is required to substantially close the interface. The fluorescence quenching in response to L-serine binding generally correlates with enzymatic inhibition, but there appears to be a slight lag in inhibition relative to quenching at low serine concentrations. The observed fluorescence quenching of residues in the regulatory domains of D-3-phosphoglycerate dehydrogenase provide the first direct evidence for a conformational change in response to effector binding and provide a means to monitor the first step in the allosteric mechanism.

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D-3-Phosphoglycerate dehydrogenase (PGDH)1 (EC 1.1.1.95) from Escherichia coli is an allosterically regulated enzyme that is inhibited by L-serine, the end product of its metabolic pathway (1, 2). The enzyme is a tetramer of identical subunits and the effect of L-serine binding is mainly on the $k_{cat}$ of the enzyme. L-Serine is bound between noncovalently associated regulatory domains of adjacent subunits (3). The homotetrameric enzyme contains two such interfaces, each of whose two domains are related by an approximate 180° axis of symmetry and each of which is capable of binding two L-serine molecules that form a hydrogen-bonded network between the adjacent domains. Mutagenesis of the residues participating in this hydrogen-bonding network results in completely active enzyme with decreased or complete loss of sensitivity to L-serine (4). A striking feature of the crystal structure of PGDH is that the serine molecules are completely buried within the closed interface and inaccessible to solvent. Earlier work (5) demonstrated that tethering adjacent regulatory domains together by engineering disulfide bonds across the interface produced an inhibited enzyme in the absence of L-serine. This inhibitory effect was reversible by reduction and could be repeated through successive cycles of oxidation and reduction of the disulfide bonds. These observations led to the hypothesis that the result of serine binding was to draw the adjacent regulatory domains together, because covalently coupling them led to a qualitatively similar result. This L-serine-induced conformational change is then translated to the active site, perhaps involving a domain shuttle mechanism (6), to produce inhibition. The apparent need for the regulatory domain interface to open and close suggested that this might be accomplished by a molecular hinge, perhaps involving the extended β-sheet formed by the union of the two domains, so that the interface opens without complete dissociation. The work reported here describes tryptophan fluorescence studies consistent with this hypothesis.

MATERIALS AND METHODS

PGDH was expressed and isolated as described previously (5, 7). Activity was determined at constant temperature in 20 mM TriA buffer at either pH 6.8 or 7.5 as indicated in the text using a-ketoglutarate as the substrate (8) and by monitoring the decrease in absorbance of NADH at 340 nm (9). Protein concentration was determined by the Bradford method (Bio-Rad, 500-0002) with a standard curve generated with bovine serum albumin (10). The accuracy of the Bradford assay for PGDH with this standard was confirmed by amino acid analysis. All mutations reported are constructed in PGDH where the four native cysteine residues in each subunit have been converted to alanine. This construct, which is referred to as PGDH$_{C}$$\alpha$ in the text, was produced for an earlier study (5) and used here for consistency with planned fluorescence resonance energy transfer studies, which will utilize an engineered cysteine residue to form an adduct with a fluorescent reporter. Kinetically, native PGDH and PGDH$_{C}$$\alpha$ are identical in $k_{cat}$ (8 s$^{-1}$) and $IC_{50}$ for serine (8–10 μM). PGDH$_{C}$$\alpha$ has a $K_{m}$ for a-ketoglutarate 5-fold higher than the native enzyme.

Mutagenesis was performed by PCR (11). All PCR reagents were obtained from Perkin-Elmer. PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc.) Restriction fragments were isolated from agarose gels with a QIAprep spin miniprep kit (Qiagen Inc.), and all mutations were confirmed by Sanger dideoxy sequencing, which was performed with the Sequenase version 2.0 sequencing kit from U. S. Biochemical Corp. PCR products were placed into plasmids by way of flanking restriction sites, and the entire length of the re-
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RESULTS

PGDH contains a single native tryptophanyl residue per subunit whose fluorescence is not affected by the presence of L-serine. This residue, Trp-139, is approximately 60 A away from the regulatory domain interface and is found in a relatively hydrophobic environment \( \lambda_{\text{max}} (335 \text{ to } 355 \text{ nm}) \) formed mainly by residues from the nucleotide binding domain of the adjacent subunit. To explore the regulatory domains, residues on its solvent-exposed side were individually converted to tryptophan. Their locations are shown schematically in Fig. 1. Four residues from the \( \alpha \)-helix bordering the interface were selected, Gly-349, Leu-351, Phe-358, and Glu-360. These mutants are listed in Tables I and II along with their fluorescent and kinetic properties. G349W, L351W, and F358W displayed a slight shift in \( \lambda_{\text{max}} \) to higher wavelength but still indicated a primarily hydrophobic environment for the predominant fluorescence, and also showed a substantial ability to be quenched by L-serine binding (Fig. 2). An additional mutant, which removed the native tryptophan from PGDH but retained the E360W mutation, E360W/W139F, was also produced to demonstrate that the quenching effect of L-serine binding was specific for the tryptophan at position 360. This is confirmed by its \( F_0/F \) ratio shown in Table I. E360W is quenched almost 2-fold, whereas E360W/W139F, containing a single tryptophan at position 360, is quenched almost 3-fold.

An additional mutant, D388W/W139F, which contains a single tryptophan exposed to solvent \( \lambda_{\text{max}} = 355 \text{ nm} \) but which resides on the second \( \alpha \)-helix over from the regulatory domain interface, was not quenched by L-serine but bound L-serine as well as E360W. The Stern-Volmer constants from solute quenching studies with sodium iodide and acrylamide demonstrate that E360W is equally accessible to both reagents but that the native Trp-139 in PGDH\(_{\text{Glu}} \) and L351W, for example, are only minimally accessible and more so for acrylamide than for iodide, which indicates that they are well shielded by surrounding structure. Inspection of the structure of PGDH indicates that L351W, and F358W, would reside deep in the crevice between regulatory domains, whereas E360W would be on the surface of the molecule. This observation is consistent with their \( \lambda_{\text{max}} \) and the solute quenching characteristics of these mutants.

Kinetically, E360W is indistinguishable from its predecessor, PGDH\(_{\text{Glu}} \), and its ability to be inhibited by L-serine binding is only very slightly decreased. The \( K_{\text{cat}} \) for E360W/W139F is increased approximately 10-fold, whereas the \( k_{\text{cat}} \) is increased 2-fold. This may reflect the fact that Trp-139 resides in a hydrophobic pocket bordering the Glu-His pair involved in catalysis and that its replacement with phenylalanine causes a perturbation of the active site. The ability of E360W, E360W/W139F, and D388W/W139F to be inhibited by L-serine is only slightly less than native, whereas that of G349W, L351W, and F358W is substantially impaired. A tryptophan at position 351 could in fact completely occlude the serine binding pocket, whereas tryptophan residues at positions 349 and 358 could shield access to the binding site to some extent. Alternatively, the presence of the excess bulk of tryptophan within the crevice itself could hinder adjacent domain association.

The crystal structure of PGDH with bound L-serine shows that L-serine is inaccessible to solvent (3–5). In order for serine to access its binding site, either the adjacent regulatory domains must separate from each other to some extent without completely dissociating, or a channel to the surface large enough to pass serine must form. In either event, the binding of serine causes a conformational change that results in its being sequestered from solvent. This proposed domain motion appears to be reflected in the L-serine-specific quenching of the tryptophan at position 360 in the mutants E360W and E360W/W139F. That this is most likely due to the effect of L-serine binding rather than to a direct collisional effect with the exposed tryptophan residue is shown in Fig. 3. L-Serine itself is unable to quench L-tryptophan in solution (Table I) and D-serine, which has the same chemical composition as L-serine but only binds minimally to PGDH because of its opposite stereochemistry, is not an effective quencher. The amount of quenching that is seen with D-serine is probably due to several factors. Amino acids in general, such as leucine, can quench to a small degree, perhaps by a collisional process or perhaps through marginal interaction with the serine binding site. D-Serine itself probably binds to PGDH with millimolar IC\(_{50}\) values similar to those for glycine and alanine (6), and the
preparation of d-serine contains approximately 1% L-serine.

If the tryptophan at position 360 is in fact being quenched by increasing proximity to the adjacent domain in response to L-serine binding, then that quenching may be due to contact with residues on the adjacent domain directly across from Trp-360. Inspection of the crystal structure shows that the side chain of Thr-352 of the adjacent domain is the closest residue to the position that would be occupied by Trp-360. In fact, simulated mutation of Glu-360 to tryptophan by molecular graphics using the crystal coordinates of the inhibited enzyme indicates that this tryptophan could be brought to within 4–5 Å of the threonine side chain. When Thr-352 is mutated to either alanine, valine, or serine, the ability of L-serine to quench Trp-360 is severely impaired (Figs. 2 and 4 and Table I). However, the

Fig. 2. Fluorescence emission spectra of PGDH mutants. Top left, fluorescence emission spectra of approximately equimolar amounts of (listed in ascending order of intensity) PGDH_{4C/A} (----), F358W (--), L351W (- - -), G349W (--- ---), and E360W (----); top right, emission spectra of PGDH_{4C/A} with increasing concentrations of L-serine from 0 to 65 µM; bottom left, emission spectra of E360W with increasing concentrations of L-serine from 0 to 60 µM; bottom right, emission spectra of E360W/T352A with increasing concentrations of L-serine from 0 to 110 µM.
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Fluorescence quenching of PGDH mutants

| PGDH          | λ_{max} (nm) | K_{SV} (μM) | K_{SV} acrylamide | F_{0}/F (L-serine) |
|---------------|-------------|-------------|-------------------|-------------------|
| PGDH_{4C/A}   | 335         | 0.4         | 3.7               | 1.00              |
| E360W/W139F   | 355         | 19          | 20                | 2.83              |
| D388W/W139F   | 355         | —           | —                 | 1.09              |
| L351W         | 340         | 0.4         | 3.7               | 1.04              |
| G349W         | 340/352     | —           | —                 | 1.01              |
| F358W         | 340         | —           | —                 | 1.00              |
| E360WT352A    | 355         | —           | —                 | 1.10              |
| E360WT352V    | 355         | —           | —                 | 1.14              |
| E360WT352S    | 355         | —           | —                 | 1.13              |
| L-Tryptophan  | 356         | 13          | —                 | 1.00              |

a Determined at the λ_{max}. The value for F is determined at 100 μM L-serine.

b Plot has downward curvature, estimated from initial slope.
c Not determined.
d The spectra exhibit a bimodal optimum of approximately equal intensity.

Kineti properties of PGDH mutants

| PGDH          | K_{cat} (μM) | k_{cat} (s^{-1}) | k_{cat}/K_m (s^{-1} μM) | IC_{50} (L-serine) |
|---------------|--------------|------------------|-------------------------|-------------------|
| PGDH_{4C/A}   | 220          | 8.1              | 3.7                     | 8                 |
| E360W         | 290          | 8.6              | 3.0                     | 32                |
| E360W/W139F   | 3300         | 15.1             | 4.6                     | 42                |
| D388W/W139F   | 8400         | 16.7             | 2.0                     | 17                |
| L351W         | 113          | 14.0             | 1.2                     | >10,000           |
| G349W         | 305          | 6.2              | 2.0                     | 1000              |
| F358W         | 255          | 14.3             | 5.6                     | 675               |
| E360WT352A    | 425          | 2.9              | 6.7                     | 1                 |
| E360WT352V    | 543          | 4                | 7.5                     | 13                |
| E360WT352S    | 425          | 11.9             | 2.8                     | 2                 |

* The value is for α-ketoglutarate and saturating NADH.

FIG. 3. Fluorescence quenching of PGDH_{4C/A} and E360W in response to specific amino acids. Normalized curves of fluorescence intensity are plotted against amino acid concentration: ●, quenching of PGDH_{4C/A} with L-serine; ■, quenching of E360W with L-serine; ▲, quenching of E360W with L-leucine; ◇, quenching of E360W with D-serine.

ability of serine to inhibit activity actually increases with these mutants (Table II). Thus, the decrease in quenching resulting from these mutations cannot be accounted for by an impairment of serine binding.

Fig. 5 shows a comparison of the fluorescence quenching, the serine inhibition, and the serine binding for E360W expressed as a percent of total. Two features of these plots stand out. The first is that there is a lag in inhibition of activity compared with fluorescence quenching at low L-serine concentrations (<30 μM). The second is that 90% or greater of both the fluorescence quenching and serine inhibition of enzyme activity have occurred by the time that only 2 serine molecules are bound per tetramer. The Scatchard plot, which is qualitatively similar to that determined for native PGDH (6), shows characteristics of positive cooperativity at low serine concentrations and a total of four serine binding sites per tetramer, which is consistent with the crystal structure of PGDH with bound serine (3).

DISCUSSION

The mutations at E360W and D388W place tryptophan residues on each of the two α-helices that run parallel to the regulatory domain interface. E360W is in the helix bordering the domain interface, whereas D388W is in the outer helix approximately 17 Å from the interface. The observation that E360W, the residue at the edge of the interface, is quenched by L-serine binding and D388W, which is removed from the interface, is not, supports the idea that quenching is a result of increased proximity between residues of the adjacent domains at their interface.

Additional data also suggest that it may be the hydroxyl oxygen of Thr-352 that is primarily responsible for quenching the fluorescence of Trp-360. Both serine and threonine possess a hydroxyl group attached to the α-carbon. In the case of threonine, an additional methyl group is also attached to the α-carbon. The crystal structure in the vicinity of Thr-352 indicates that the β-methyl group restricts rotation about the Ca–Cβ bond, because it would collide with the backbone oxygen of Gly-349. Serine, on the other hand, would be free to rotate because of the lack of the β-methyl group. In fact, a rotation of 90° of the serine hydroxyl would allow it to hydrogen bond with the ε-amino group of Lys-356, effectively removing it from proximity to Trp-360. Valine, which contains two methyl groups on the β-carbon, would also be restricted in its rotational freedom like threonine but would place a methyl group in approximately the position of the hydroxyl group found in threonine. That this quenching is a function of L-serine concentration, that serine does not directly quench Trp-360, and that...
The effect of L-serine on fluorescence quenching and activity and L-serine binding to E360W. **Top:** ●, percent of total quenching versus L-serine concentration; ■, percent inhibition versus L-serine concentration; □, percent occupancy of L-serine binding sites per tetramer versus total L-serine concentration. **Bottom:** Scatchard plot of L-serine binding to E360W. r, moles of L-serine bound per moles of protein (tetramer); F, free L-serine concentration.

Trp-360 and Thr-352 are found directly across from each other on opposite subunits is consistent with the model that proposes that the consequences of serine binding is to draw the adjacent domains together. However, these data by themselves still do not sufficiently distinguish that possibility from others that may entail a more complex rearrangement of atoms such as might be caused by the closing of a channel from the surface to the interior of the domain when serine binds. Nevertheless, the data do support the conclusion that there is interaction across the noncovalent interface and that this interaction is influenced by serine binding. The simplest way for this to be accomplished would be for the β-sheet to act as a hinge and flex slightly. Although there is little precedent for this in structural biology, other interpretations would require a much greater degree of internal structural rearrangement of the regulatory domains. The results cannot simply be explained by a global change in the tryptophan environment triggered by a mutation at position 352, because the quantum yields of E360W and E360W/T352A are approximately the same in the absence of L-serine.

Comparison of the binding of L-serine to PGDH with the fluorescence quenching and activity inhibition data shows that inhibition and quenching are largely complete with less than full occupancy of the serine binding sites. Although the inhibition and quenching curves generally follow each other, the sigmoidal shape of the plot for inhibition is more evident than for quenching. If one assumes that the two regulatory domain interfaces in the PGDH tetramer are both functional and equally capable of binding serine, an occupancy of two serine molecules per tetramer suggests that each interface contains on the average of one bound serine. This indicates that the presence of one serine at each interface is sufficient to inhibit the enzyme to greater than 90% and is accountable for most of the tryptophan quenching that is observed. These data imply that binding of serine to the third and fourth sites in the tetramer, whereas perhaps having some small additional effect, is largely unnecessary. These implications are consistent within the context of the model that proposes that the interface closes by drawing together domains that are relatively rigid. Thus, the binding of a single serine at each interface is sufficient to close the interface. Binding of serine at one site may in fact exclude the binding of a second molecule of serine at the same interface except at higher concentrations of serine when perhaps both sites have a higher probability of being occupied simultaneously. It is interesting to note that the Scatchard plot displays elements of both positive and negative cooperativity. This may be consistent with selective binding to one site per interface at low concentrations and binding to the second site at each interface only at very high concentrations because of steric constraints for the second site induced by the closing of the interface in response to occupancy of the first site. Alternatively, the observed stoichiometry of serine binding could be satisfied if one of the two interfaces is essentially unable to bind serine at low concentrations. This would imply, however, that binding at a single interface is sufficient to inhibit all four active sites unless only two active sites are functional in the first place. The present data are not sufficient to distinguish between these possibilities.

Cooperativity in serine binding would also be expected to manifest itself in the tryptophan quenching curve if one were directly a consequence of the other. Perhaps additional conformational changes in the linkage between the serine binding sites and the active sites are occurring that are not revealed by the tryptophan quenching data. Whereas serine binding and fluorescence quenching are taking place within the same domain, catalysis occurs at a more remote location and there are two catalytic sites for each regulatory domain interface in the tetrameric molecule. Perhaps the linkage of the regulatory sites to the catalytic sites are not equivalent and this is reflected in the inexact correlation between inhibition and quenching.

In combination with the earlier observation that covalently tethering the adjacent domains together (5) results in inhibition of activity in the absence of serine, these data are consistent with the model that the domains move relative to one another at the interface as relatively rigid bodies. If serine binding draws adjacent regulatory domains together, it implies that the intermolecular distances between residues on each domain changes substantially. The magnitude of the shift between a locus on one subunit relative to the other subunit may be able to be determined by fluorescence resonance energy transfer analysis after the introduction of a second fluorophore. However, the flexing of the β-sheet, if any, will probably only be clearly visualized by determining the crystal structure of the uninhibited enzyme, but obtaining suitable crystals of the uninhibited enzyme has proven problematic. Nonetheless, the construction of tryptophan mutants that respond to serine binding provide the first direct observation that serine binding produces significant conformational changes in the regulatory domains of PGDH, which is the first step in the dynamics of the allosteric process.

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REFERENCES
1. Sugimoto, E., and Pizer, L. I. (1968) J. Biol. Chem. 243, 2090–2098
2. Sugimoto, E., and Pizer, L. I. (1968) J. Biol. Chem. 243, 2081–2089
3. Schuller, D., Grant, G. A., and Banaszak, L. (1995) Nat. Struct. Biol. 2, 69–76
4. Al-Rabiee, R., Zhang, Y., and Grant, G. A. (1996) J. Biol. Chem. 271, 23235–23238
5. Al-Rabiee, R., Lee, E. J., and Grant, G. A. (1996) J. Biol. Chem. 271, 13013–13017
6. Grant, G. A., Schuller, D. J., and Banaszak, L. J. (1996) Protein Sci. 5, 34–41
7. Schuller, D. J., Fetter, C. H., Banaszak, L. J., and Grant, G. A. (1989) J. Biol. Chem. 264, 2645–2648
8. Zhao, G., and Winkler, M. E. (1996) J. Bacteriol. 178, 232–239
9. Tobey, K. L., and Grant, G. A. (1986) J. Biol. Chem. 261, 12179–12183
10. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
11. Cormack, B. (1991) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 8.5.1–8.5.9, John Wiley and Sons, New York
12. Eftink, M. R., and Ghiron, C. A. (1981) Anal. Biochem. 114, 199–227
13. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950