The Contribution of Adjacent Subunits to the Active Sites of D-3-Phosphoglycerate Dehydrogenase*

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D-3-Phosphoglycerate dehydrogenase (PGDH) from *Escherichia coli* is allosterically inhibited by L-serine, the end product of its metabolic pathway. Previous results have shown that inhibition by serine has a large effect on $V_{\text{max}}$ and only a small or negligible effect on $K_m$. PGDH is thus classified as a V-type allosteric enzyme. In this study, the active site of PGDH has been studied by site-directed mutagenesis to assess the role of certain residues in substrate binding and catalysis. These consist of a group of cationic residues (Arg-240, Arg-60, Arg-62, Lys-39, and Lys-141) that potentially form an electrostatic environment for the binding of the negatively charged substrate, as well as the only tryptophan residue found in PGDH and which fits into a hydrophobic pocket immediately adjacent to the active site histidine residue. Interestingly, Trp-139 and Lys-141 are part of the polypeptide chain of the subunit that is adjacent to the active site. The results of mutating these residues show that Arg-240, Arg-60, Arg-62, and Lys-141 play distinct roles in the binding of the substrate to the active site. Mutants of Trp-139 show that this residue may play a role in stabilizing the catalytic center of the enzyme. Furthermore, these mutants appear to have a significant effect on the cooperativity of serine inhibition and suggest a possible role for Trp-139 in the cooperative interactions between subunits.

D-3-Phosphoglycerate dehydrogenase (PGDH)† (EC 1.1.1.95) from *Escherichia coli* is a homotetrameric enzyme that is inhibited in an allosteric manner by L-serine (1, 2). The crystal structure (Ref. 3, Protein Data Bank, Brookhaven National Laboratory, code 1PSD) shows that each subunit is composed of three domains which are referred to as the substrate binding domain, the nucleotide binding domain, and the regulatory or serine binding domain. Each subunit interacts noncovalently with two adjacent subunits through contacts at their respective regulatory domains and their respective nucleotide binding domains (Fig. 1). L-Serine binds in the two interfaces formed between each pair of regulatory domains while the catalytically active site is in a cleft between the substrate binding domain and the nucleotide binding domain of each subunit. Serine binding induces a conformational change at the regulatory domain interfaces (4, 5) that is subsequently transferred to the active sites to produce inhibition of catalytic activity. Moreover, the inhibition of catalytic activity by serine displays sigmoidal kinetics, indicating a cooperative effect between subunits. The nature of this cooperativity has not yet been established, but serine binding data (4) suggest that binding of serine to only two sites, of a total of four serine binding sites, is all that is necessary to inhibit the enzyme by more than ninety percent.

Inspection of the active site of PGDH from the crystal structure (3) of the inhibited enzyme (with L-serine bound) reveals several unique features that may be related to substrate binding and catalysis and perhaps the allosteric interaction between subunits. First, the active site of PGDH contains five positively charged residues whose side chains protrude into the solvent accessible space of the active site cleft. These are Lys-39, Arg-60, Arg-62, Arg-240, and Lys-141. Because the substrate, phosphoglyceric acid, is a very negatively charged molecule, these positively charged residues may play a role in substrate binding. Interestingly, one of these basic residues, Lys-141, is contributed by the adjacent subunit (3). That is, by the subunit adjacent to the subunit that contains the active site in question (Fig. 2). The charged groups of the side chains of both Lys-141 and Arg-60 are very near the binding site for the negatively charged heavy metal compounds that were used for multiple isomorphous replacement phasing in the crystallographic studies. This is thought to be the most probable binding site for the phosphate group of the substrate.

Second, the Glu-His pair, which makes up the so-called charge relay system common to many dehydrogenases and similar to that found in serine proteases as a His-Asp pair, rests at the top of a hydrophobic pocket into which is inserted Trp-139 of the adjacent subunit (Fig. 3). The suggestion has been made that residues at the active site that come from the subunit adjacent to that which forms that particular active site cleft may play a role in the cooperative nature of the inhibition kinetics. Preliminary evidence suggests that cooperativity is observed for both serine binding and for inhibition kinetics but that they are not identical. This manuscript explores the active site of PGDH by site-directed mutagenesis and presents data that may have implications for subunit cooperativity in inhibition of catalytic activity.

MATERIALS AND METHODS

PGDH was expressed and isolated as described previously (6, 7). Activity was determined at a constant temperature in 20 mM Tris buffer at pH 7.5 using either α-ketoglutarate (8) or hydroxypyruvic acid phosphate (1, 2) as the substrate and by monitoring the decrease in absorbance of NADH at 340 nm (9). Protein concentration was determined by the Bradford method as described previously (4, 10). All mutations are constructed in PGDH<sub>4C/A</sub> which is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine. This construct has been described previously (4) and is used here for consistency of comparison to past studies. Kinetically, native PGDH and PGDH<sub>4C/A</sub> are very similar (4).

Mutagenesis was performed by PCR (11). All PCR reagents were available on line at http://www.jbc.org. © 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
FIG. 1. Diagram of the arrangement of subunits in the tetramer of PGDH derived from the crystal structure (3). The two depictions are rotated 90 degrees to the plane of the paper relative to each other. The four identical subunits are designated A, B, C, and D, and the location of the regulatory domain (Reg), substrate binding domain (Sub), and nucleotide binding domain (Nuc) are shown for subunit 9. The active site clefts are designated by arrows, and the interfaces where serine binds are indicated with asterisks. The approximate location of Trp-139 and Lys-141 are shown with black dots.

RESULTS AND DISCUSSION

Description of the Active Site—The reaction catalyzed by PGDH involves hydride transfer and proton extraction at the C2 position of the substrate, interconverting phosphohydroxypyruvate and phosphoglycerate. This is mediated by NADH and His-292 (Fig. 2). In addition, Glu-269 acts in tandem with His-292 to form a proton shuttle as seen in many dehydrogenases and in serine proteases as a His-Asp pair (Fig. 2). Also common to dehydrogenases such as malate and lactate dehydrogenase, which have similar substrates and which share this type of mechanism, is a basic residue that serves to anchor the C1 carboxyl group. Arg-240 in PGDH appears to correspond to this residue based on its position relative to His-292 and NADH (Fig. 2). The additional basic residues, Arg-60, Arg-62, Lys-39, and Lys-141′, are unique to PGDH and may interact with the acidic group at the distal end of the substrate. The natural substrate for PGDH is phosphohydroxypyruvate, but α-ketoglutarate has also been found to be an effective substrate (5). Thus, this group would be a phosphate for phosphohydroxypyruvate and a carboxylate for α-ketoglutarate. The distance along the carbon chain between the two acidic groups in each substrate is nearly identical.

Arg-60 and Lys-141′ are in closest proximity to His-292 with their protonatable groups in approximately the same plane as those of His-292 and Arg-240 (Fig. 2). In addition, the distance between Arg-240 and the cationic groups of Lys-141′ and Arg-60 is approximately the same as the distance between the two anionic groups of the substrates. Arg-62 and Lys-39 are further removed, with Lys-39 being the furthest from Arg-240 and nearest the opening of the active site cleft. Thus, it appears from the structure that Arg-240, Arg-60, Lys-39, and possibly Arg-62 are the most likely residues to interact with the substrate when it is bound at the active site. One must keep in mind, however, that the available structure is from the inhibited enzyme with bound L-serine. Although it is known that the substrate can bind to the inhibited enzyme, it is not known how serine inhibition affects the relative position of residues in the active site cleft.

Interestingly, Lys-141′ is contributed by the adjacent subunit whose nucleotide binding domain overlaps with the active site cleft of its partner (Fig. 1). In addition to Lys-141′, Trp-139′ also projects into the active site cleft of its adjacent subunit. In this case, however, Trp-139′ is not solvent-exposed but rather fits into a hydrophobic pocket formed by residues of the adjacent subunit like a tab into a slot (Fig. 3). This pocket is made...
up largely of proline and phenylalanine residues that surround Trp-139. Curiously, the His-Glu pair, which includes the active site histidine, is immediately adjacent to the aromatic ring of Trp-139 and appears to form the top of the pocket. This intimate association of active site catalytic residues with a hydrophobic residue from the adjacent subunit is curious, and its unique positioning suggests that it could play a role in stabilizing the structure of the active site.

Mutations of the Active Site Cationic Residues—The five cationic residues found in the active site cleft of PGDH likely make up a pre-organized electrostatic framework for the binding of the doubly negative-charged substrate. However, the spatial arrangement of these residues suggested that they may not all play a significant role in substrate binding. Thus, each residue was mutated in turn to an alanine side chain to assess the relative role of their cationic groups. The kinetic parameters...
ters determined for the mutant enzymes with either α-ketoglutarate or phosphohydroxypyruvate as substrate are presented in Table I. Data was determined for both substrates to assess if the additional oxygen function found on the phosphate group of phosphohydroxypyruvate demonstrated a differential interaction with one or more of the cationic residues.

These data indicate that the major effect of the phosphate group of phosphohydroxypyruvate compared with the carboxyl group of α-ketoglutarate appears to be in the $K_m$, which shows a 15-fold difference for PGDH$_{CA}$, whereas the $k_{cat}$ appears largely unaffected. This relationship generally holds for the mutants as well. However, the difference in $K_m$ is smaller with some of the mutants, particularly for R240A, where only an approximately 2-fold difference is seen. This might initially suggest that the side chain of Arg-240 is involved in interacting with the phosphate but not the carboxylate. However, the enzyme crystal structure shows that Arg-240 is positioned very close to His-292 and the nicotinamide ring of NADH. This would indicate that Arg-240 interacts with the anionic group closest to the catalytic center of the substrate which is the carboxyl at C-1 in both substrates. In fact, the structure suggests that this is the only residue available for binding the C-1 end of the substrate to the active site. Thus, a more probable interpretation is that Arg-240 is critical for binding of either substrate, and its absence essentially overrides any incremental binding effect that may be contributed differentially by other cationic side chains binding with the phosphate or the carboxylate.

Mutation of Lys-39, which is the furthest removed from the catalytic center, has little effect on the $K_m$ and only a 2-fold effect on the $k_{cat}$. Lys-39 would thus seem not to play a significant role in the interaction with the substrate or in the catalysis. Excepting Arg-240 for the reason discussed above, the most pronounced effect in $k_{cat}$ is seen for R60A and K141A, whereas the most pronounced effect on $K_m$ is seen for R62A. In addition, the difference in $K_m$ for R62A and K141A between the two substrates is about half of what it is for R60A. Taking the structure of the substrate and the geometry of these residues in the active site together with these data, they suggest that Arg-60, Arg-62, and Lys-141 all play a role in binding the end of the substrate furthest from the active center and that Arg-62 and Lys-141’ may interact a little more strongly with the phosphate group of phosphohydroxypyruvate than does Arg-60. Interestingly, the most pronounced effect overall, again excluding Arg-240, is seen for Lys-141’, whose $k_{cat}/K_m$ ratio is 2 orders of magnitude less than PGDH$_{CA}$ and at least an order of magnitude less than R60A and R62A. This represents a major contribution to the active site from a residue that comes from the adjacent subunit.

The effect of the cationic residue mutations on the ability of serine to inhibit the enzyme is shown in Table I, which lists the concentration of serine at which 50% inhibition is achieved and the coefficient of cooperativity for inhibition. The coefficient of cooperativity for inhibition of native PGDH is approximately 1.7. This suggests that at least two of the subunits interact cooperatively in the inhibition. This is consistent with the preliminary observation that serine binding to only two of the four sites is required for inhibition. Furthermore, the symmetry of the tetramer suggests that subunit dimers may be the functional allosteric units. While Lys-141’ seems to increase the enzyme’s sensitivity to serine, it does it to no greater extent than do the other cationic mutants, and its coefficient of cooperativity is also essentially unchanged from the unmutated form. Thus, although Lys-141’ contributes an electrostatic ligand to the adjacent subunit in PGDH and is critical for activity, it does not seem to be involved in the subunit cooperativity seen in the serine inhibition kinetics because its absence does not reduce the apparent cooperativity.

**Mutations at Tryptophan 139’—Like Lys-141’, Trp-139’ also appears to interact with the active site of the adjacent subunit. However, in this case it does so by filling a hydrophobic pocket at the base of two catalytically important residues, His-292 and Glu-269. The role of Trp-139’ was investigated by mutating it to residues with hydrophobic side chains of decreasing length and volume. The kinetic parameters for these mutations are listed in Table I. All mutations tested lower the $k_{cat}/K_m$ with the largest effect being on the $k_{cat}$ of the enzyme. Fig. 4 shows the relationship between the $k_{cat}/K_m$ of these enzymes and the volume of the residue at position 139’. Substitution of tryptophan with phenylalanine is the most conservative and lowers the $k_{cat}/K_m$ by approximately an order of magnitude. Substitution with leucine decreases this parameter by more than 2

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**TABLE I**

| PGDH               | $K_m$ ($\mu M$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1} \mu M$) | IC$_{50}$ ($\mu M$) | $n_A$ |
|--------------------|-----------------|----------------------|-------------------------------|---------------------|-------|
| α-Ketoglutarate as substrate: | | | | | |
| PGDH$_{CA}$       | 0.63            | 31.3                 | $5.0 \times 10^4$             | 10                  | 1.67  |
| K39A              | 0.55            | 14.2                 | $2.6 \times 10^4$             | 10                  | 1.71  |
| R62A              | 2.3             | 11.8                 | $5.1 \times 10^3$             | 6                   | 1.72  |
| R60A              | 0.95            | 0.95                 | $1.0 \times 10^3$             | 6                   | 1.78  |
| K141A             | 1.0             | 0.33                 | $3.3 \times 10^2$             | 5                   | 1.79  |
| R240A             | 3.3             | 0.16                 | $4.8 \times 10^1$             | 7                   | 1.70  |
| W139              | 0.63            | 31.3                 | $5.0 \times 10^4$             | 10                  | 1.67  |
| W139F             | 1.6             | 5.5                  | $5.2 \times 10^3$             | 15                  | 1.51  |
| W139L             | 1.1             | 0.1                  | $9.3 \times 10^1$             | 28                  | 1.21  |
| W139V             | 1.8             | 0.045                | $2.5 \times 10^1$             | 19                  | 1.34  |
| W139A             | 3.2             | 0.116                | $3.7 \times 10^1$             | 26                  | 1.33  |
| W139G             | 3.3             | 0.055                | $1.6 \times 10^1$             | 12                  | 1.23  |

- Determined in 20 mM Tris, pH 7.5.
- The value is determined with saturating NADH.
- The value for native PGDH is the same.

**Phosphohydroxypyruvate as substrate:**

| PGDH$_{CA}$       | 0.042           | 31.3                 | $7.5 \times 10^5$             | 105                 | 1.67  |
| K39A              | 0.040           | 15.2                 | $3.8 \times 10^5$             | 101                 | 1.23  |
| R62A              | 0.3             | 13.9                 | $4.6 \times 10^4$             | 10                  | 1.67  |
| R60A              | 0.043           | 0.51                 | $1.2 \times 10^4$             | 28                  | 1.21  |
| K141A             | 0.11            | 0.22                 | $2.0 \times 10^3$             | 28                  | 1.21  |
| R240A             | 1.5             | 0.11                 | $7.3 \times 10^1$             | 12                  | 1.23  |
orders of magnitude, and W139V, W139A, and W139G are over 3 orders of magnitude lower. The decrease in $k_{cat}/K_m$ is approximately linear with respect to the volume of the side chain until a threshold is reached for the valine mutant. Smaller side chains produce very little additional effect. This suggests that the bulk of Trp-139 is necessary for stabilization of the pocket and thus the relationship of the His-Glu pair to each other and to the active site. As the volume of the side chain decreases, the pocket may become more and more unstable until a point is reached where the maximum effect is seen. Additional reduction in volume past this point no longer produces an additional effect on the activity.

Unlike Lys-141, mutation of Trp-139 appears to have a definite effect on the cooperativity of serine inhibition. Fig. 5 shows the serine inhibition data determined for Trp-139 mutants fitted to the Hill equation, and the coefficients of cooperativity determined from the fit are listed in Table I. The curve for W139F appears to retain a sigmoidal character similar to that for the unmutated enzyme, but the coefficient of cooperativity is somewhat lower than that for the unmutated enzyme. The inhibition curves for the other mutants all appear to be more hyperbolic in nature, and their coefficients are significantly reduced. It is possible that perhaps the leucine and valine side chains are causing a steric problem because their side chains might not fit as smoothly into the pocket as the aromatic residues. However, this is not the case for glycine, which has no side chain to get in the way. Yet, it produces the lowest degree of cooperativity of any of the mutants while maintaining an IC$_{50}$ very close to that of unmutated enzyme. This data is consistent with the possibility that Trp-139 may, at least in part, contribute to the interaction between subunits and the mechanism of inhibition. The relatively larger effect of these mutations on $k_{cat}$ as compared with $K_m$ is also consistent with PGDH being a V-type enzyme. These data suggest that only a relatively small movement of Trp-139 away from the His-Glu pair would be enough to produce the desired effect and would be consistent with relatively small, subtle changes in conformation caused by serine binding. While this may be a rather simplified view, with the actual mechanism being more complex and involving other induced conformational changes at the active site, these studies provide the first suggestion of a possible molecular basis for the effect on the PGDH active site of effector binding to a remote location.

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. Grant, G. A., and Xu, X. L. (1998) J. Biol. Chem. 273, 22389–22394
5. Grant, G. A., Schuller, D. J., and Banaszak, L. J. (1996) Protein Sci. 5, 34–41
6. Al-Rahiee, R., Lee, E. J., and Grant, G. A. (1996) J. Biol. Chem. 271, 13013–13017
7. Schuller, D. J., Feiter, 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. (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. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
13. Creighton, T. E. (1984) Proteins: Structures and Molecular Properties, p. 7, W. H. Freeman and Company, New York