Specific Interactions at the Regulatory Domain-Substrate Binding Domain Interface Influence the Cooperativity of Inhibition and Effector Binding in *Escherichia coli* d-3-Phosphoglycerate Dehydrogenase*

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The crystal structure of d-3-phosphoglycerate dehydrogenase reveals a limited number of contacts between the regulatory and substrate binding domains of each subunit in the tetrameric enzyme. These occur between the side chains of Arg-339, Arg-405, and Arg-407 in the regulatory domain and main chain carbonyls in the substrate binding domain. In addition, Arg-339 participates in a hydrogen bonding network within the regulatory domain involving Arg-338 and Tyr-410, the C-terminal residue of the enzyme subunit. Mutagenic analysis of these residues produce profound effects on the enzyme’s sensitivity to serine, the cooperativity of serine inhibition, and in some cases, the apparent overall conformation of the enzyme. Mutations of Arg-405 and Arg-407, which span the interface where the two domains come together, reduce the cooperativity of inhibition and increase the sensitivity of the enzyme to serine concentration. Serine binding studies with Arg-407 converted to Ala demonstrate that cooperativity of serine binding is also significantly reduced in a manner similar to the reduction in the cooperativity of inhibition. Mutations of Tyr-410 and Arg-338 decrease the sensitivity to serine without an appreciable effect on the cooperativity of inhibition. In the case of Tyr-410, a deletion mutant demonstrates that this effect is due to the loss of the C-terminal carboxyl group rather than the tyrosine side chain. All mutations of Arg-339, with the exception of its conversion to Lys, had profound effects on the stability of the enzyme. In general, those mutants that decrease sensitivity to serine are those that participate mainly in intradomain interactions and may also directly affect the serine binding sites themselves. Those mutants that decrease cooperativity are those that participate in interdomain interaction within the subunit. The observation that the mutants that decrease cooperativity also increase sensitivity to serine suggests a potential separation of pathways between how the simple act of serine binding results in noncooperative active site inhibition in the first place and how serine binding also leads to cooperativity between sites in the native enzyme.

**MATERIALS AND METHODS**

Mutants of PGDH were produced, expressed in *E. coli*, and isolated as described previously (6, 7). Catalytic activity was determined at 25 °C in 20 mM Tris buffer at pH 7.5 using α-ketoglutarate as the substrate and by monitoring the decrease in absorbance of NADH at 340 nm (8, 9). Protein concentration was determined by the Bradford method (10, 11) and by quantitative amino acid analysis for the equilibrium dialysis binding experiments. All mutations are constructed in PGDH*<sub>4C/A</sub>* to maintain consistency with previous studies (5, 6, 10, 12). PGDH*<sub>4C/A</sub>* is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine (10). The IC<sub>50</sub> value for L-serine is that concentration of serine that produces a 50% inhibition of the enzyme activity. Kinetic parameters were determined using direct linear plots (13). Enzyme homogeneity was judged by SDS gels. Figs. 1–4 were produced with Molscript (14).

The oligomeric association state of the mutants was monitored by intrinsic fluorescence and serine binding. Subunit dissociation results in a shift in fluorescence from 340 to 360 nm (10, 15) and a loss of serine binding (16). All mutants in this study that were recovered with activity maintained an emission maximum at 340 nm and retained their ability to bind serine, indicating an intact association of subunits.

* The abbreviation used is: PGDH, phosphoglycerate dehydrogenase.

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Equilibrium dialysis was performed in 500-μl dialysis cartridges obtained from Sialomed, Inc. (Columbia, MD). Dialysis was performed for 16 h with L-[3H]serine as a tracer in appropriate concentrations of unlabeled l-serine. Cells were sampled in triplicate, and the average of 10-min counts were used to calculate concentrations of free and bound l-serine. The nominal PGDH concentration was 5 μM tetramer in all binding experiments.

Serine inhibition plots were fit to the Hill equation (12) (Equation 1),

\[ Y = \frac{[L]/K_d + [L]^n}{K_d + [L]^n} \]  

and serine binding data were fit to the Adair equation (12) (Equation 2),

\[ Y = \frac{(L/K_1) + (2L^2/K_2K_3) + (3L^3/K_2K_3K_4) + (4L^4/K_2K_3K_4K_5)}{4(L/K_1) + (L/K_2K_3) + (L/K_2K_3K_4) + (L/K_2K_3K_4K_5)} \]

or the equation for independent binding sites (Equation 3),

\[ r = \frac{[L]/K_d + [L]}{K_d + [L]} \]

using Kaleidograph (Synergy Software). Y is the fractional occupancy or inhibition, \( r \) is mol of ligand bound per mol of acceptor, \( p \) is the number of binding sites, and \( n \) is the Hill coefficient. Intrinsic site dissociation constants were calculated from the Adair constants by using the following statistical relationships for a molecule where \( n \) sites are occupied at maximal binding and where \( K'_n \) are the intrinsic dissociation constants (17), \( n = 2 \) sites: \( K'_1 = 2K_1, K'_2 = K_2/2; n = 3 \) sites: \( K'_1 = 3K_1, K'_2 = K_2/3; n = 4 \) sites: \( K'_1 = 4K_1, K'_2 = 3K_2/2, K'_3 = 2K_3/3, K'_4 = K_4/4 \).

**RESULTS**

Description of the Regulatory Domain-Substrate Binding Domain Interface—Fig. 1 shows an α-carbon chain diagram of a subunit of PGDH. The nucleotide binding domain (bottom) is linked to the regulatory domain (top) by way of a helix that extends along the body of the substrate binding domain (shaded dark). The regulatory and substrate binding domains are covalently linked by a single strand of polypeptide, and the substrate and nucleotide binding domains are covalently linked by two strands of polypeptide. The only apparent interaction between the regulatory and substrate binding domains are hydrogen bonds between the guanidino groups of Arg-339, Arg-405, and Arg-407 on the regulatory domain and main chain carbonyl groups on the substrate binding domain (Figs. 2 and 3). Specifically, the guanidino group of Arg-339 forms hydrogen bonds with the main chain carbonyls of Leu-332 and Asn-318, the guanidino group of Arg-407 forms a hydrogen bond with the main chain carbonyl of Ser-316, and the guanidino group of Arg-405 forms a hydrogen bond with the main chain carbonyl of Arg-97 and Leu-76. In addition, Arg-339 participates in a hydrogen bonding network with Tyr-410 (the C-terminal residue of the protein), Arg-338, Glu-387, and Ala-385 (Fig. 4). Specif-
ically, the ring hydroxyl of Tyr-410 is within hydrogen bonding distance of the guanidino group of Arg-339 and the main chain carbonyl of Gly-336. The C-terminal carboxyl of Tyr-410 is within hydrogen bonding distance of the guanidino group and the main chain amino group of Arg-338. The guanidino group of Arg-338 is also within hydrogen bonding distance of the side chain carboxyl of Glu-387 and the main chain carbonyl of Ala-385. This network, along with the interaction of Arg-339 with Leu-332 and Asn-318, completes a bridge across the loop containing Gly-336 and Gly-337 (Figs. 2 and 3), which has been shown to be functional in transmitting the effect of serine binding to the active site (5).

Site-directed Mutagenesis—The arginyl residues that span the interface, Arg-405, -407, and -339, were converted to alanine individually (Table I). R405A and R407A both had a principle effect on the $k_{cat}$ of the enzyme, which resulted in a lowering of the $k_{cat}$ and $k_{cat}/K_m$ values by 1 and 2 orders of magnitude, respectively. In addition, both mutations produced an enzyme that was more sensitive to serine and that displayed a significant decrease in the Hill coefficient for cooperativity of inhibition. The double mutant, R405A/R407A, appeared to have a profound effect on the structure of the enzyme, resulting in the disruption of the nucleotide binding site as judged by the protein’s inability to bind to the 5'-AMP affinity column. Activity and serine sensitivity were restored by incorporating more polar residues at these sites (R405N/R407N), but the loss in cooperativity of serine inhibition remained.

R339A and R339N yielded low levels of protein that lacked sufficient activity to be accurately measured. On the other hand, R339K produced protein in good yield with a 10-fold reduction in $k_{cat}$, a small decrease in serine sensitivity, and a reduced Hill coefficient. Conversion of Arg-338 to Gly or removal of Tyr-410 (DY410) from the C terminus decreased serine sensitivity by 40- and 20-fold, respectively, without a major effect on the $k_{cat}/K_m$ or the Hill coefficient. Y410A displayed kinetic parameters similar to that of DY410 and with a modest increase in serine sensitivity. E387A showed little effect on any of the measured parameters.

PRINCIPAL EXPERIMENTAL RESULTS

The mutant that showed the greatest effect on the Hill coefficient, R407A, was analyzed for its ability to bind serine by equilibrium dialysis. The data were fit to the Adair equation for cooperative sites (Fig. 5) as well as to the equation for equivalent, independent sites (Fig. 6). The Adair equation was fit for four binding sites whereas the equation for equivalent sites was fit for two total sites. The latter was necessary because the equation for independent sites, unlike the Adair equation, does not accommodate an analysis with less than total site occupancy. Visual as well as statistical analysis ($\chi^2$ value) indicates that the best fit is produced by the Adair equation, which can
accommodate sites that are not independent. Both analyses indicate that there is a significant decrease in the intrinsic dissociation constant for the first site (Table II). The Adair fit also indicates that there is still a low level of positive cooperativity for binding to the second site. This is consistent with an upward concavity at the beginning of the Scatchard plot (not shown).

**DISCUSSION**

Mutation of the arginyl residues spanning the regulatory domain-substrate binding domain interface of PGDH produced profound effects on its sensitivity to serine, the cooperativity of serine inhibition, and in some cases, the apparent overall conformation of the enzyme. Conversion of Arg-405 and Arg-407 to alanine, individually, produced similar effects that differed mainly in the extent of the change. Although the values for $k_{cat}$ were reduced by 1 and 2 orders of magnitude, respectively, a substantial decrease in the Hill coefficient was also produced especially for R407A. Serine binding measurements on R407A demonstrated that only two of the four sites are occupied and that there is a substantial decrease in positive cooperativity among these sites. The lack of binding of the last two sites is similar to that seen with the native enzyme. It has been proposed (12) that occupancy of one site at the binding interface closes the interface and precludes binding of the second molecule within the same interface. That this appears to occur with greater ease with this mutant is consistent with the notion that eliminating the interaction mediated by the side chain of Arg-407 may ease a conformational restraint so that the interface can close more easily in response to the first ligand.

The fit to the Adair plot seems to be better than to that for a rectangular hyperbola for independent sites, both by inspection and by consideration of the values for $\chi^2$. The positive cooperativity of binding is also significantly reduced by this mutation. Thus, although serine is still an effective inhibitor, the degree of interaction between subunits appears to be diminished, and the apparent reduction in positive cooperativity is consistent with a significantly reduced Hill coefficient. In addition, the observation that overall sensitivity to serine concentration increases while cooperativity decreases reinforces the previous conclusion (12) that simple inhibition (noncooperative) is modulated at the level of the individual subunit.

The double mutant, R405A/R407A, was inactive and incapable of binding to the 5'-AMP column through its NAD binding site. Presumably, the protein was not globally denatured because it appeared to be relatively stable after synthesis. Interestingly, the double mutant with asparagine replacing alanine (R405N/R407N) restored structural integrity while basically

**TABLE I**

| Mutant     | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ µM$^{-1}$) | IC$_{50}$ Ser (µM) | Hill coefficient ($n_l$) |
|------------|------------|----------------------|-----------------------------------|-------------------|-------------------------|
| 4C/A       | 0.6        | 31.3                 | $5.0 \times 10^4$                 | 10                | 2.02 ± 0.07             |
| R405A      | 1.7        | 5                    | $2.9 \times 10^3$                 | 3                 | 1.42 ± 0.09             |
| R407A      | 1.75       | 0.6                  | $3.3 \times 10^2$                 | 4                 | 1.21 ± 0.08             |
| R405A/R407A| 0.05       | 3.4                  | $5.3 \times 10^3$                 | 2                 | 1.28 ± 0.09             |
| R339A      | 0.65       | 3.4                  | $3.3 \times 10^3$                 | ND$^a$            |                         |
| R339N      | 0.5        | 3.6                  | $7.2 \times 10^3$                 | 32                | 1.60 ± 0.06             |
| R339K      | 0.15       | 9.3                  | $6.2 \times 10^2$                 | 420               | 1.99 ± 0.09             |
| 1Y410      | 0.35       | 15.7                 | $4.5 \times 10^3$                 | 200               | 1.78 ± 0.08             |
| Y410A      | 0.15       | 9.6                  | $6.0 \times 10^0$                 | 3.5               | 1.63 ± 0.05             |
| E387A      | 0.23       | 13                   | $5.7 \times 10^1$                 | 11                | 2.26 ± 0.11             |
| 1Y410/E387A| 0.2        | 12.2                 | $6.1 \times 10^0$                 | 200               | 1.84 ± 0.10             |

$^a$ Did not bind to 5'-AMP column. Enhanced protein band present in lysate but no activity detected.

$^b$ Bound to 5'-AMP column but at very low yield and activity, too low to determine kinetic parameters.

**FIG. 5. Binding of L-serine to R407A PGDH.** The data are plotted as fractional occupancy of sites ($Y$) versus free serine concentration ($\mu$M). The solid line is the fit of the data to the Adair equation for a molecule with four sites. The top panel shows the full range of ligand concentration, and the bottom panel expands the plot at low ligand concentrations.
**Specific Subunit Interactions Influence Cooperativity in PGDH**

Binding data are expressed as dissociation constants. Adair constants are denoted as $K_i$, and intrinsic site dissociation constants are denoted as $K_a$.

| PGDH$_{E44A}$ Adair equation | PGDH R407A Adair equation |
|------------------------------|--------------------------|
| $\mu M$                      | $\mu M$                  |
| $K_1$                        | $53.3 \pm (1.3 \times 10^{-4})$ | $9.1 \pm (1 \times 10^{-3})$ |
| $K_2$                        | $2.8 \pm (2.6 \times 10^{-3})$ | $11.3 \pm (8 \times 10^{-4})$ |
| $K_3$                        | $83.9 \pm (5.3 \times 10^{-4})$ | Very large |
| $K_4$                        | Very large               | Very large |
| $R_a$                        | $160$                    | $36$     |
| $K_a$                        | $156$                    | $11$     |
| $R_a$                        | $3$                      | $6$      |
| $K_a$                        | $28$                     | Very large |
| $R_a$                        | Very large               | Very large |
| $R_a$                        | $0.99553$                | $0.98862$ |
| $\chi^2$                     | $0.00693$                | $0.02938$ |
| $\chi^2$                     | $18$                     | $11$     |

- Fitting statistics: $R$ is the correlation coefficient and $\chi^2$ represents the accumulated deviation between the data and the calculated curves.

- FIG. 6. Binding of l-serine to R407A PGDH. The data are plotted as mol of ligand bound per mol of tetrameric protein ($r$) versus free serine concentration ($\mu M$). The solid line is the fit to the equation for two independent sites. The top panel shows the full range of ligand concentration, and the bottom panel expands the plot at low ligand concentrations.

- Reflecting the properties of the individual alanine mutants, R405A and R407A. The asparagine side chains may have restored hydrogen bonding capability although they are considerably shorter than the native arginyl side chains. On the other hand, the increased bulk and polarity of the asparaginyl side chains over that of alanine may have provided critical stability in the polypeptide folding process perhaps by providing alternative interactions. Nonetheless, even though catalytic activity was mostly restored, cooperativity was still significantly decreased.

- Mutation of Arg-339 to Ala (R339A) produced a protein that bound to the 5’-AMP column but was recovered at such a low level that it was not possible to do a satisfactory kinetic analysis. This property could not be overcome by substituting asparagine for alanine (R339N), but could be reversed to a large extent by lysine (R339K). Because the native arginyl residue forms hydrogen bonds with main chain carbonyls, the length of the side chain for hydrogen bonding rather than the presence of a formal positive charge appears to be critical in this case. The hydrogen bonding network observed between Arg-339, Tyr-410, Arg-338, and Glu-387 appears to be critical for serine sensitivity but not so much for cooperativity or kinetic activity. Mutation of the Arg-338 side chain or complete removal of Tyr-410 significantly decreased the enzyme’s sensitivity to serine. However, a similar result was not seen when the tyrosine side chain was changed to an alanine. If anything, the enzyme became more sensitive. This indicates that the tyrosine side chain itself is not required unless perhaps it plays a small role in fine tuning the position of Arg-339, but rather the C-terminal carboxyl at that position is critical.

- Removal of the Glu-387 side chain produced little effect, either by itself or when the C-terminal carboxyl was also missing (compare E387A to ΔY410/E387A and ΔY410). These results point to the Arg-338 side chain as being the critical element. It may possibly serve to stabilize the orientation of Arg-339 through its effect on the rotational freedom of the polypeptide chain or by holding the polypeptide chain translationally steady at this point.

- In general, those mutants that decreased cooperativity are also those that participate in interdomain interaction within the subunit. Those mutants that show their greatest effect on sensitivity to serine are those that participate only in intradomain interactions. What is more curious is the observation that mutants that presumably decrease interaction across the domain interface not only decrease cooperativity but also increase the sensitivity of the enzyme’s active site to inhibition by serine. This suggests a potential separation of pathways between how the simple act of serine binding results in noncooperative active site inhibition in the first place and how serine binding also leads to cooperativity between sites. The question remains as to the identity of the interactions that govern noncooperative inhibition, because disrupting the known interactions seems to increase the potency of serine. The explanation probably lies in the fact that we do not have a picture of the conformation that occurs in the inhibited enzyme because it has not been possible to produce a stable crystal under these circumstances. Thus, there may be additional interactions that occur in the absence of effectors that are not present in the inhibited state that account for this situation. Alternatively, these interface mutations themselves may induce additional interactions at the interface or in the global orientation of the domains that enhance the effect of serine. In view of the lack of a crystal structure of the active enzyme, further probing by specific mutagenesis of this domain interface will be necessary to address these questions.
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Specific Subunit Interactions Influence Cooperativity in PGDH