The Mechanism of Velocity Modulated Allosteric Regulation in D-3-Phosphoglycerate Dehydrogenase

SITE-DIRECTED MUTAGENESIS OF EFFECTOR BINDING SITE RESIDUES

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Regina Al-Rabiee, Yueping Zhang, and Gregory A. Grant‡

From the Department of Molecular Biology and Pharmacology and the Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

D-3-Phosphoglycerate dehydrogenase (EC 1.1.1.95) from Escherichia coli catalyzes the first committed step in serine biosynthesis and is allosterically regulated by L-serine, the end product of the pathway. Each subunit of the homotetramer is made up of three distinct domains with one of the intersubunit contacts being between adjacent regulatory domains. Each regulatory domain interface contains two symmetrical serine binding sites such that serine forms hydrogen bonds to both domains across the interface. Previous work (Al-Rabiee, R., Lee, E. J., and Grant, G. A. (1996) J. Biol. Chem. 271, 13013–13017) demonstrated that when adjacent regulatory domains are covalently linked to one another by engineered disulfide bonds, the enzyme was inactivated. Breaking the disulfide bonds by reduction restored enzymatic activity. This study demonstrates that the complementary situation is also true. Site-directed mutagenesis of three residues at the effector binding site, His344, Asn346, and Asn364, render the enzyme increasingly less susceptible to inhibition by the effector. When mutations result in a situation where it is no longer possible to establish a stable hydrogen bonding network across the regulatory domain interface, the inhibitory capacity of the effector is lost. Furthermore, mutations that produce as much as 5 orders of magnitude decrease in the ability of L-serine to inhibit the enzyme have no appreciable effect on the $K_m$ or $k_{cat}$ of the enzyme. These observations support the model that predicts that catalytic activity in D-3-phosphoglycerate dehydrogenase is regulated by the movement of adjacent regulatory domains about a flexible hinge and that effector binding tethers the regulatory domains together producing a state that results in a stable, open active site cleft that is no longer able to promote catalysis.

D-3-Phosphoglycerate dehydrogenase (PGDH)† (EC 1.1.1.95) from Escherichia coli is a homotetrameric enzyme whose subunits are composed of three distinct domains (1). PGDH is inhibited by the end product of its pathway, L-serine, with the regulation being of the $V_{max}$-type (2, 3). The serine binding sites are formed by the noncovalent contact between two adjacent regulatory domains with two symmetrical serine binding sites at each of the two regulatory domain interfaces in the tetramer. Because serine forms hydrogen bonds to adjacent domains across this interface, this arrangement is likely to be the basis for the inhibitory effector modulation of PGDH (1, 4) (see Fig. 1).

A model of allosteric regulation in PGDH has been developed (4) and predicts that catalysis and allosteric inhibition of PGDH by L-serine are implemented through the motion of rigid domains about flexible hinges. It has been suggested that one of these hinges is located at the noncovalent interface between two adjacent regulatory domains and that serine binding tethers the adjacent regulatory domains to each other so that they cannot move about their hinge. (4). When serine is bound, it is buried in the interface such that it is no longer accessible to solvent. It appears that the interface between regulatory domains must open to some extent in order for serine to associate and dissociate from the enzyme. Because PGDH remains a tetramer in the active as well as inhibited state, this opening does not involve complete subunit dissociation, although partial dissociation of the interface would seem to be needed. The model predicts that relaxation of subunit association about this hinge, which would be the case when the effector is not bound, releases the substrate binding domain from its contacts with the regulatory domain so that the substrate domain is now free to close the active site cleft for catalysis to occur.

The crystal structure of PGDH determined with L-serine bound to the effector site (1) suggests that the side chains of three amino acids participate in hydrogen bond formation with the carboxyl and amino group of serine. In addition, two additional hydrogen bonds appear to form with the serine hydroxyl group through water molecules that interact with the main chain carbonyl oxygens of two additional amino acids (see Fig. 1). This hydrogen bonding network appears to act as a tether between adjacent regulatory domains because three of the involved protein amino acids are found in one domain and the other two are found in the adjacent domain. Thus, a three-point contact of the protein with the three functional groups of serine appears to form the basis for serine binding and allosteric inhibition.

Additional evidence supporting the role of the effector molecule in tethering the adjacent regulatory domains together was acquired when it was shown (5) that PGDH could be completely and reversibly inhibited in the absence of effector by covalently cross-linking adjacent domains with engineered cysteine residues that formed disulfide bonds between domains. This report provides additional experimental evidence for the model of allosteric inhibition of PGDH demonstrating the converse situation. That is, specific mutation of residues at the regulatory domain interface directly affects the ability of the effector molecule to inhibit the enzyme and mutations that result in the loss of the ability of effector ligands to cross-link.
adjacent domains result in the complete loss of inhibitory capacity for those ligands.

**MATERIALS AND METHODS**

PGDH was expressed, isolated, and assayed as described previously (5–8). Kinetic parameters were determined in the usual manner from either double reciprocal plots of 1/v versus 1/S or direct plots of S/v versus S. Substrate concentration was altered in the presence of saturating NADH, and K was determined by performing the analysis in the presence of varying amounts of L-serine.

Agarose, isopropyl-1-thio-β-D-galactopyranoside, ampicillin, polyethyleneimine, imidazole, α-ketoglutarate, and NADH were from Sigma. Restriction enzymes were from Boehringer Mannheim or New England Biolabs, and T4 DNA ligase was purchased from Boehringer Mannheim.

Mutants were produced by polymerase chain reaction mutagenesis using standard procedures (9), 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. All polymerase chain reaction reagents were obtained from Perkin-Elmer, and restriction fragments were isolated from agarose gels with the Gene Clean kit from Bio 101.

The structure of PGDH was viewed with a Silicon Graphics IRIS molecular graphics system using SYBYL (Tripos Inc.) software, and the SYBYL BIOPOLYMER program was used to simulate mutagenesis.

**RESULTS AND DISCUSSION**

The crystal structure of PGDH indicates that the effector binding site residues whose side chains interact with the effector, L-serine, are His344, Asn346, and Asn364 (Fig. 1). These were converted to alanine residues, and the effect of the mutation on the ability of L-serine to inhibit enzyme activity was measured. In addition to single mutations, three sets of double mutations were also performed. Because the serine hydroxyl group interacts with main chain atoms through water molecules, it was not possible to study this interaction by mutation. However, by using glycine as the effector ligand, the effects of the other mutations in the absence of serine hydroxyl interaction could be assessed. These results are presented in Table I.

L-serine, with three interacting functional groups, displays inhibitory capacity for all three single residue mutants but with IC values 2–4 orders of magnitude higher than for the native enzyme. With the exception of the serine hydroxyl interaction, which is potentially present with all three single mutants, the stronger interaction appears to involve the Asn”, Asn364, serine α-amino group triad because the Asn to Ala mutants both display weaker interaction as judged by the IC than does the His to Ala mutant. This observation is not surprising in view of the potential for an additional hydrogen bond occurring between the two Asn residues at the interface that would contribute to the tethering of adjacent subunits. This interaction appears to be capable of forming a triangular hydrogen bonding network with this triad that serves to stabilize the enzyme. In terms of the contribution of the two Asn residues, the interaction with Asn” appears to contribute the most in regard to maintaining the interface association. Again this is not surprising because Asn” is found on the domain adjacent to that which contains His344 and Asn346 and potentially contributes two hydrogen bonds across the interface.

All three double mutants, which represent the three possible combinations of two residue mutations, are no longer capable of being inhibited by L-serine. In all three cases, the only potential hydrogen bond reaching across to the opposite domain involves a serine hydroxyl-water interaction (with Thr352 in the case of the His→Ala mutant and with Val353 in the case of the other two double mutants). The Asn”→Ala single mutant, which is marginally inhibited by serine, displays a similar situation involving Val353. The difference in inhibition between this mutant and the double mutants may reflect the presence of both Asn346 and His344, which may serve to orient the serine molecule for a more stable interaction of the hydroxyl group with the enzyme in the single residue mutant. The complete lack of inhibition of the double mutants reflects the fact that when the potential hydrogen bonding interaction across the interface is diminished sufficiently, the effector is no longer able to exert its effect.

This observation is confirmed with glycine inhibition. When at least two of the nonhydroxyl hydrogen bonding interactions are maintained such that they span the domain interface, as in the case of His→Ala and Asn→Ala, some degree of inhibition is retained. However, when all potential hydrogen bonding across the interface is eliminated, as in Asn”→Ala, the ability of glycine to inhibit is abolished.

The inhibition pattern seen with L-alanine, which unlike

![Figure 1](http://www.jbc.org/) Depiction of the effector binding site of PGDH with bound L-serine based on the crystal structure coordinates. Potential hydrogen bond interactions are indicated with dashed lines, and the distances are given in angstroms. The prime is used to indicate residues that reside on the adjacent subunit relative to those without primes. The identity of the atoms are indicated with the shading of the spheres. Dark shading indicates oxygen, medium shading indicates carbon, and no shading indicates nitrogen. With the exception of the two water molecules, hydrogen atoms are not shown. In this case the hydrogens are depicted by the unshaded spheres. Diagrams were produced with MOLSCRIPT (11).
glycine possesses a chiral $\alpha$-carbon atom like that in $\text{l}$-serine, reinforces the observations made with glycine. The 3-fold decrease in inhibition seen with Asn$^{346} \rightarrow \text{Ala}$ may reflect the difficulty of the hydrophobic methyl side chain to occupy the water-filled hydrophilic pocket normally occupied by the serine hydroxyl. The inhibition pattern of $\beta$-alanine reflects a similar situation where the increased distance between the amino and carboxyl group due to the additional methylene carbon precludes productive hydrogen bonding across the interface except in the case of His$^{344} \rightarrow \text{Ala}$ where the Asn$^{346}, \text{Asn}^{346}, \alpha$-amino group triad is capable of forming. However, this interaction is 1–2 orders of magnitude less than that seen with either glycine or $\text{l}$-alanine, possibly because of the additional bulk of the extended molecule in the binding pocket. Previous studies (4) have shown that other amino acids are capable of inhibiting PGDH at millimolar concentrations. These include $\text{l}$-cysteine, $\text{l}$-threonine, $\text{l}$-allothreonine, $\text{l}$-homoserine, and $\text{l}$-$\alpha$-aminobutyric acid. $\text{l}$-Norvaline, $\text{l}$-norleucine, $\text{l}$-glutamine, and $\text{l}$-phenylalanine do not show appreciable inhibitory action. So although the ability of the effector to inhibit activity varies by as much as 5 orders of magnitude in response to these mutations, $K_m$ and $k_{\text{cat}}$ are essentially unaltered. Thus, when serine is present in the native enzyme, it shuts down the catalytic mechanism through a long range interaction (the effector and catalytic sites are 33 Å apart). The outcome of mutation of His$^{344}, \text{Asn}^{346}, $ and Asn$^{346}$ supports their role in the mechanism of allosteric regulation, but alteration of these residues does not irreversibly affect the integrity of the catalytic site. Taken together, these data suggest the involvement of an additional element in this process, which links serine binding to catalytic activity. The model (4) predicts that this is the interaction of the substrate binding domain with the regulatory domain and that this is directly modulated by effector binding.

When considered in combination with the disulfide cross-linking study (5) discussed earlier, these data provide strong evidence to support the hypothesis that allosteric inhibition of PGDH is mediated by the degree of interaction between two regulatory domains of adjacent subunits of the enzyme and that this association is mediated by the effector molecule. Because it has previously been shown that subunit dissociation of the tetrameric enzyme does not occur in either the active or inhibited state (10) and that serine is completely buried in the interface and not accessible to solvent (1, 4), the two adjacent regulatory domains must reversibly open to some extent at the interface without breaking the eight membered $\beta$-sheet, which forms the noncovalent association between adjacent domains. This is consistent with the model (1, 4) that allosteric inhibition of PGDH involves a series of domain movements about flexible

| TABLE I | Inhibition of native and mutant PGDH |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | 1-Serine | Glycine | $\text{l}$-Alanine | $\alpha$-Alanine | $(R)(-)$-2-Amino-1-propanol | $(S)(+)$-2-Amino-1-propanol | 3-Hydroxy propionic acid | Ethanolamine |
| Native          | 0.008    | 1.8     | 5            | 165             | $>250$             | $>250$            | $>250$          | $>250$          |
| H344A           | 0.8      | 14      | 6.5          | 175             | $>250$             | $>250$            | $>250$          | $>250$          |
| N346A           | 6        | 32      | 92           | $>250$          | $>250$             | $>250$            | $>250$          | $>250$          |
| N346A/N346$^b$  | $>250$   | $>250$ | $>250$       | $>250$          | $>250$             | $>250$            | $>250$          | $>250$          |
| N346A/H344$^b$  | $>250$   | $>250$ | $>250$       | $>250$          | $>250$             | $>250$            | $>250$          | $>250$          |
| H344A/N364$^b$  | $>250$   | $>250$ | $>250$       | $>250$          | $>250$             | $>250$            | $>250$          | $>250$          |

$^a$ Concentration that produces 50% inhibition.
$^b$ The prime (') indicates that Asn$^{346}$ resides on the subunit adjacent to that which contains His$^{344}$ and Asn$^{346}$.

| TABLE II | Comparison of the steady state kinetic and inhibition parameters of native and mutant PGDH |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | $K_m$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ | $IC_{50}$ | $K_m$ | $IC_{50}$ | $K_m$ |
|                 | mM   | s$^{-1}$ | s$^{-1}$M$^{-1}$ | mM    | s$^{-1}$ | s$^{-1}$M$^{-1}$ | mM  |
| Native          | 0.042 | 7.7     | $1.8 \times 10^{-5}$ | 0.005  | 0.008  | 0.005        |
| N346A           | 0.044 | 19.2    | $4.4 \times 10^{-5}$ | 4      | 6      | ND$^*$       |
| H344A/N364$^b$  | 0.038 | 8.2     | $2.2 \times 10^{-5}$ | NA$^*$ | >250    | NA$^*$       |

$^a$ $K_m$ and $k_{\text{cat}}$ are determined for the reduction of $\alpha$-ketoglutarate in the absence of inhibitor.
$^b$ Determined by varying substrate concentration at different levels of $\text{l}$-serine.
$^c$ Determined in the presence of saturating substrate and cofactor.
$^d$ Determined by equilibrium dialysis (4).
$^e$ ND, not determined; NA, not applicable.
hinges. The movement of the regulatory domains relative to each other has been likened to that of a “piano hinge” because the data suggest that the hinging may take place along a relatively long axis coincident with the length of the domain interface. This is inferred from the observation that two molecules of serine bind at opposite ends of the interface. At this time, however, the location and nature of the hinge movement is not known. Additional work is in progress to identify the hinge location and to determine the manner in which this action is transferred to the active site to result in the inhibition of enzyme activity.

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