The decrease in sensitivity was caused primarily by a decrease in the atomic coordinates and structure factors (code 2P9E, 2P9C, 2PA3, and 2P9G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The atomic coordinates and structure factors (code 2P9E, 2P9C, 2PA3, and 2P9G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

From the Departments of 9 Molecular Biology and Pharmacology and 8 Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 and the 9 Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843

† This work was supported by National Institutes of Health Grant GM 56676 (to G. A. G.) and by the Robert A. Welch Foundation (to J. C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The abbreviations used are: PGDH, phosphoglycerate dehydrogenase; NBD, nucleotide binding domain; PEG, polyethylene glycol; r.m.s.d., root mean square deviation; LLG, log likelihood gain; TLS, translation libration screw.

Received for publication, February 7, 2007, and in revised form, March 29, 2007 Published, JBC Papers in Press, April 24, 2007, DOI 10.1074/jbc.M701174200

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

The Effect of Hinge Mutations on Effector Binding and Domain Rotation in Escherichia coli D-3-Phosphoglycerate Dehydrogenase*

Sanghamitra Dey§, Zhiqin Hu§, Xiao Lan Xu§, James C. Sacchettini§, and Gregory A. Grant†§

D-3-Phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95) from Escherichia coli contains two Gly-Gly sequences that have been shown previously to have the characteristics of hinge regions. One of these, Gly336-Gly337, is found in the loop between the substrate binding domain and the regulatory domain. Changing these glycine residues to valine affected the sensitivity of the enzyme to inhibition by L-serine but not the extent of inhibition. The decrease in sensitivity was caused primarily by a decrease in the affinity of the enzyme for L-serine. These mutations also affected the domain rotation of the subunits in response to L-serine binding. A major conclusion of this study was that it defines a minimal limit on the necessary conformational changes leading to inhibition of enzyme activity. That is, some of the conformational differences seen in the native enzyme upon L-serine binding are not critical for inhibition, whereas others are maintained and may play important roles in inhibition and cooperativity. The structure of G336V demonstrates that the minimal effect of L-serine binding leading to inhibition of enzyme activity requires a domain rotation of approximately only 6° in just two of the four subunits of the enzyme that are oriented diagonally across from each other in the tetramer. Moreover the structures show that both pairs of Asn190 to Asn190 hydrogen bonds across the subunit interfaces are necessary for activity. These observations are consistent with the half-the-sites activity, flip-flop mechanism proposed for this and other similar enzymes and suggest that the Asn190 hydrogen bonds may function in the conformational transition between alternate half-the-site active forms of the enzyme.

D-3-Phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95) from Escherichia coli is a tetrameric enzyme that displays cooper-
Is its principal effect on the binding of serine to the effector site or is it on the transduction of the effect of $\beta$-serine binding to the active site?

In this study, ligand binding analyses demonstrated that the characteristics of serine binding are significantly altered in the G336V and G336V,G337V mutant enzymes. Gel permeation chromatography and analytical ultracentrifugation demonstrated that this is not due to changes in subunit aggregation. Modeling of the relationship of serine binding to inhibition of activity demonstrated that the inhibition characteristics of the mutant enzyme can be accounted for mainly by the change in ligand binding dynamics. In addition, the crystal structures of these mutant enzymes demonstrate that previously observed domain rotations are not required for inhibition of activity by serine to occur.

**MATERIALS AND METHODS**

PGDH was expressed in *E. coli* and purified using 5’-AMP-Sepharose affinity chromatography (11, 12). Enzyme activity was determined by the change in absorbance at 340 nm due to the conversion of NADH to NAD$^+$ at pH 7.5 (13) using α-ketoglutarate as the substrate (14). All PGDH constructs were based on PGDH 4CA where the four native cysteinyl residues are converted to alanyl residues (15). PGDH 4CA was chosen to be consistent with previous studies and so that new cysteines can eventually be introduced for the incorporation of reporter groups. For simplicity, this construct will be assumed as the background in all subsequent descriptions and not be referred to specifically.

**Serine Binding Analysis**—Serine binding was measured by equilibrium dialysis in 200-μl dialysis chambers (Sialomed, Inc., Columbia, MD) for 16 h with 1-[$^3$H]serine in appropriate concentrations of unlabeled 1-serine. Cells were sampled in triplicate, and the averages of 10-min counts were used to calculate concentrations of free and bound 1-serine. The nominal PGDH concentration was $5–10 \text{ mM}$ tetramer, and all binding was performed in the presence of $100 \text{ mM}$ NADH. Serine binding data were fit to the Adair equation for four sites (Equation 1) (16) using Kaleidograph (Synergy Software) as described previously (17, 18).

$$Y = \left[\frac{\left(4[L]/K_1\right)}{1 + \left(4[L]/K_1\right)}\right] + \left[\frac{\left(12[L]^2/K_1^2 K_2\right)}{1 + \left(2[L]/K_1\right)}\right] + \left[\frac{\left(12[L]^3/K_1^3 K_3\right)}{1 + \left(L/K_1\right)}\right] + \left[\frac{\left(6[L]^4/K_1^4 K_4\right)}{1 + \left(L/K_1\right)}\right] + \left[\frac{\left(4[L]^4/K_1^4 K_4\right)}{1 + \left(L/K_1\right)}\right]$$

Data were also analyzed using a Hill plot for the graphical determination of the first dissociation constant (18). Protein concentration was determined by quantitative amino acid analysis.

**Inhibition Analysis**—Serine inhibition plots were fit to the Hill equation (16, 18)

$$I = \left[\frac{[L]}{\left(I_{0.5}\right)^n} + \left[L\right]^n\right]$$

where $I$ is the fractional inhibition, $L$ is the concentration of ligand, $n$ is the Hill coefficient, and $I_{0.5}$ is the inhibitor concentration at half-maximal inhibition.

**Gel Permeation Chromatography**—Gel permeation chromatography was performed in a 2.6 × 90-cm column of Sephacryl S-300 HR operated at a flow rate of $\sim 0.35 \text{ ml/min}$ in 20 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA. The elution buffer for samples run in the presence of serine contained 0.2 mM 1-serine. 2-min fractions were collected, and the absorbance was read at 280 nm.

**Analytical Ultracentrifugation**—Sedimentation velocity and sedimentation equilibrium analyses were performed by Alliance Protein Laboratories (Camarillo, CA). Sedimentation equilibrium analysis was performed by loading samples of protein at concentrations of 2.33, 1.17, 0.777, 0.388, 0.194, 0.117, 0.0388, and 0.0194 mg/ml into three six-channel centerpieces and centrifugation at 8,000 rpm at 25 °C in a Beckman Optima XL-A analytical ultracentrifuge. Optical scans were recorded at wavelengths of 300, 280, or 230 nm after 16, 20, and 24 h. The rotor speed was then increased to 20,000 rpm for 12 h to clear the meniscus region, and a last scan was taken to measure the baseline offset for each sample. The resulting data were analyzed by non-linear least square techniques using the program KDALTON (19, 20). A solvent density of 1.0094 g/ml and a partial specific volume of 0.7455 ml/g at 25 °C were calculated using the program SEDNTERP (21). Sedimentation velocity analyses were performed by loading protein at 1.5 mg/ml into two-channel charcoal–Epon cells with 3-mm path length and at 0.375 mg/ml in a similar cell with 12-mm path length. Buffer was loaded into the reference channel of each cell, and the samples were loaded into a Beckman Optima XL-A analytical ultracentrifuge. The rotor was brought to 3000 rpm, and the samples were scanned to confirm proper sample loading. The rotor speed was increased to 40,000 rpm for the duration of the run. Scans were taken approximately every 4 min for 4 h. The data were analyzed with the programs SEDFIT (22) and SVEDBERG (23, 24).

**Crystallization and Data Collection**—Crystallization conditions were broadly screened using Crystal screen, Crystal screen II, and PEG/Ion screen from Hampton Research and Wizard I and II from Emerald Biosystems. All crystallization trials contained 5 mM NADH. Approximately 1000 crystallization trials were completed for each mutant and complex. Crystals were obtained for the serine-free G336V mutant protein, the G336V mutant with 5 mM serine, and the G336V,G337V mutant with 5 mM serine. The serine-free G336V mutant was crystallized in 1.5 M ammonium sulfate and 0.1 M sodium citrate, pH 5.5 (Wizard I & 8, Emerald Biosystems). There were two different crystal forms of the serine-bound G336V mutant. Form A was crystallized in 20% PEG 1000, 0.1 M sodium/potassium phosphate (pH 6.2), 0.2 M NaCl (Wizard II 14), and form B was crystallized in 10% PEG 3000, 0.1 M cacodylate, pH 6.5, 0.2 M MgCl$_2$ (Wizard II 27). The serine-bound double mutant G336V,G337V was crystallized in 30% PEG 400, 0.1 M acetate, pH 4.5, 0.2 M calcium acetate (Wizard I 44). The data sets for the G336V PGDH crystal and form A crystal of G336V + serine were collected at 19ID and 23ID beamlines, respectively (Advanced Photon Source, Argonne National Laboratory, Chicago, IL). Data sets for form B of serine-bound G336V and G336V,G337V were collected in house using a Rigaku Raxis IV ++ detector. All of the crystallizations were performed by the hanging drop vapor diffusion technique at 18 °C.
**Effect of Hinge Mutations on E. coli PGDH**

**TABLE 1**

Data collection, refinement, and geometry statistics

|                | G336V | G336V form A | G336V form B | G336V,G337V |
|----------------|-------|--------------|--------------|-------------|
| Data collection|       |              |              |             |
| Space group    | P4    | P6,22        | P2,2         | P2,2        |
| Unit cell dimensions |       |              |              |             |
| a = b =γ = 90° | 90°   | 90°          | 90°          | 90°         |
| c = 132.24 Å  | 132.24 Å | 133.09 Å   | 132.24 Å    | 132.24 Å    |
| c = 132.24 Å  | 132.24 Å | 133.09 Å   | 132.24 Å    | 132.24 Å    |
| a = b = γ = 90° | 90°   | 90°          | 90°          | 90°         |
| Molecules per ASU² | 1     | 1            | 2            | 2           |
| Wavelength (Å) | 0.979 | 0.979        | 1.541        | 1.541       |
| Resolution range (Å) | 35–2.6 | 30–2.6 | 35–2.6 | 35–2.6 |
| Highest resolution bin (Å) | 2.69–1.26 | 2.84–2.74 | 2.56–2.5 | 2.95–2.8 |
| Observed reflections | 1,149,410 | 618,111 | 234,844 | 84,334 |
| Unique reflections | 121,230 | 30,270 | 31,001 | 25,289 |
| Completeness (%) | 99.9 (96.1) | 99.9 (99.8) | 88.7 (72.2) | 99.5 (100) |
| Average redundancy | 18.7 (10.8) | 20.4 (16.4) | 7.6 (8.2) | 3.3 (3.3) |
| R/| 0.017 | 0.001 | 0.011 | 0.017 |
| Rsym | 1.92 | 1.81 | 1.4 | 1.87 |

Refinement statistics (REFMAC)

|                | G336V | G336V form A | G336V form B | G336V,G337V |
|----------------|-------|--------------|--------------|-------------|
| Free R value (%) | 28    | 25           | 28           | 25          |
| R value (%) | 21    | 22           | 22           | 20          |
| No. of protein residues | 1,619 | 406 | 810 | 808 |
| No. of water molecules | 77    | 18           | 54           | 49          |
| r.m.s.d. bond length (Å) | 0.017 | 0.006 | 0.011 | 0.017 |
| r.m.s.d. bond angles (°) | 1.92  | 1.81 | 1.4 | 1.87 |

Ramachandran plot (PROCHECK)

|                | G336V | G336V form A | G336V form B | G336V,G337V |
|----------------|-------|--------------|--------------|-------------|
| Most favored region (%) | 1,217 (86.3) | 311 (87.9) | 618 (87.5) | 603 (85.4) |
| Additional allowed regions (%) | 185 (13.1) | 41 (13.6) | 87 (12.3) | 98 (13.9) |
| Generously allowed regions (%) | 9 (0.6) | 2 (0.6) | 1 (0.1) | 5 (0.7) |
| Disallowed regions (%) | 0    | 0            | 0            | 0           |

¹ Asymmetric unit.
² Values in parentheses are for the highest resolution bin.
³ R = \( \sqrt{\|F_{obs}\|^2 - \|F_{calc}\|^2} / \|F_{calc}\| \) where \( F_{obs} \) is the observed intensity and \( F_{calc} \) is the average intensity of multiple observations of symmetry-related reflections.

---

**Data Processing and Structure Determination**—The data set for the G336V + serine form A crystal was processed and scaled using HKL2000 (25). The crystal was in space group P6₁,22 with one molecule in the asymmetric unit, having a high solvent content of 80%. The structure was solved by molecular replacement using Phaser (26) with the chain B of serine-bound native enzyme as the ensemble (Protein Data Bank code 1PSD) with an LLG of 3521 (Z-score of 16.42 and 59.12 for the rotation and translation function, respectively).

The serine-free mutant structure was also processed and scaled using HKL2000, and the structure was determined using Phaser with the form A mutant structure as the model (LLG = 1452); the space group was P4₃ with four molecules in the asymmetric unit.

The data set for form B of the serine-bound G336V mutant and the serine-bound G336V,G337V mutant were both indexed using LABELIT (27) and further processed and scaled using mosflm (28) and SCALA (29), respectively. The space group was P₂₁,2₂ for both the crystals with two molecules in the asymmetric unit. The structure of form B was determined using molecular replacement with the form A mutant structure as the model with an LLG of 2645. The inhibited double mutant structure was solved using Protein Data Bank code 1PSD as the model with an LLG of 4786. The geometry of these structures was largely improved using simulated annealing refinement in CNS (Crystallography and NMR System) during the early stages of model building (30). Iterative rounds of model building were performed using Xtalview (31) and Coot (32) with the Shake & wARP (33) bias-minimized electron density maps. All of the models were refined using REFMAC (34), and the serine-bound G336V (P₂₁,2₂) structure was TLS-restrained in the final stage of refinement. The quality of the models was assessed using PROCHECK (35). No side chains are in the disallowed region of the Ramachandran plot. The overall statistics are provided in Table 1. The domain movements were analyzed manually as well as using the program DynDom (36), which determines the hinge axes and the residues involved in hinge bending. For this analysis, the default settings of DynDom were used.

**RESULTS**

Previous kinetics analyses of *E. coli* PGDH (10) showed that of the two amino acid residues Gly³³⁶ and Gly³³⁷, mutations of Gly³³⁶ had a greater effect on the serine sensitivity of the enzyme and that the double mutation had a more than additive effect. Therefore, the L-serine binding characteristics of these two mutant proteins were investigated in more detail, and their crystal structures were determined in the presence and absence of L-serine (crystals were obtained only in the presence of L-serine for the double mutant protein).

**Serine Binding and Inhibition**—The results of L-serine binding analysis of G336V is shown in Fig. 1. The serine binding and inhibition profiles for G336V are compared with those for the native enzyme in Fig. 2. Although serine inhibition could be demonstrated kinetically for the G336V,G337V double mutant, the binding affinity of serine was too low to measure by equilibrium dialysis. Fitting of the data to the Adair equation for four sites yielded the intrinsic dissociation constants presented in Table 2. However, the fitting of four variables simultaneously produced relatively large errors for these values. Therefore, the data were also analyzed with a Hill plot (shown in Fig. 1B). The Hill plot allowed a graphical determination of the first dissoci-
ation constant that was used to constrain the fit of the Adair equation. When this was done, the errors for the determination of the other three dissociation constants were significantly reduced. These data indicate that mutation of these two glycyl residues to valyl residues produced a substantial increase in the cooperativity of effector binding in the G336V mutant. This increase in cooperativity manifested itself principally by a large increase in the dissociation constant for binding of the first effector molecule but also had an effect on subsequent dissociation constants.

Quaternary Structure—The increase in cooperativity seen with these mutations could be caused by conformational constraints introduced into the PGDH tetramer, or they could be caused by an alteration in the subunit aggregation. The association state of the proteins was assessed with gel permeation chromatography and analytical ultracentrifugation. Both the native and mutant proteins eluted with essentially the same volume in the presence and absence of serine (not shown) indicating that the oligomeric state of the mutant proteins was unchanged relative to the native protein even in the presence of L-serine.

Sedimentation velocity analysis of G336V yielded a sedimentation coefficient of 7.65 S that was unchanged in the presence of L-serine. This agrees very well with a sedimentation coefficient of 7.68 S determined from hydrodynamic bead modeling from the x-ray crystal structure of the native enzyme and the value of 7.85 determined previously for the native enzyme (37). A fit of the data to the program SVEDBERG also yielded a diffusion coefficient of 4.56. The S/D ratio implies a molecular mass of ~161 kDa, confirming the assignment of this species as a tetramer. Sedimentation equilibrium data were also consistent with a tetramer of 150–160 kDa.

Binding-Inhibition Linkage—Previous studies (9) have shown that the relationship between effector binding and inhibition of activity in PGDH can be represented by the relationship

\[
I = 1.33P_1 + 0.67P_2 + P_3 + P_4 \tag{Eq. 3}
\]

where \(P_i\) represents the population of enzyme species with \(i\) bound ligands. Because inhibition is dependent only on the binding of the first two ligands, the dissociation constants for the formation of \(P_3\) and \(P_4\) are relevant only in so far as they reflect the population of species with ligand bound at the first two sites. Therefore, for the purpose of modeling the linkage between binding and inhibition, the dissociation constants for the last two ligands are set equal to that of the second ligand. Fig. 3 shows that the inhibition profile can be modeled very accurately by Equation 3 using dissociation constants derived from the ligand binding analysis of G336V PGDH.

Mutant Enzyme Structure—The crystal structure of the G336V mutant enzyme was determined to 2.6-Å resolution. It crystallizes in the same space group as the native enzyme (P4\(\_3\)_2); both contain four molecules in the asymmetric unit, which also corresponds to the biological unit. The overall conformation of the enzyme is similar to the native enzyme. The two structures overlay with an r.m.s.d. of 0.33 Å for C\(_{\alpha}\) atoms. This structure has citrate, phosphates/sulfates, and cofactor NADH bound at the active site, but the density for citrate is visible only in the active sites of subunits B and C. In subunits A and D, the active sites are occupied by phosphate ions and water molecules. The citrate molecule seems to form hydrogen bonds with Arg\(^{60}\) and Lys\(^{141}\) (\(^{\prime}\) indicates a residue from the adjacent subunit). In subunits B and C, although citrate occupies a position similar to that of the natural substrate at the active site, it is still significantly distant from the catalytic dyad His\(^{292}\), Glu\(^{389}\) as well as
Effect of Hinge Mutations on E. coli PGDH

The NADH, Arg<sup>240</sup>, which usually forms hydrogen bonds with the carboxylate group of the substrate, is facing away from the citrate. In all four subunits of the tetramer, Arg<sup>240</sup> is also interacting with a phosphate ion present at the active site. There are also additional electron densities found away from the active site that are consistent with citrate molecules that are present in the crystallization buffer.

A major difference between G336V and native PGDH is evident at the point of mutation where significant alterations in the φ and ψ angles of residues His<sup>335</sup>–Gly<sup>337</sup> are observed along with the transition of residue 336 in the range of 1.8–2.6 Å in all four chains. No significant change is seen at the serine binding sites themselves between G336V and native PGDH. These observations suggest that the altered structure observed at the site of mutation is principally responsible for the altered serine binding behavior, although they are quite distant from the serine binding site itself.

In the native enzyme, Asn<sup>190</sup> of the nucleotide binding domain (NBD) appears to make hydrogen bond contact with its counterpart in the diagonally opposite subunit through a water molecule in subunits A and C and directly in subunits B and D. This novel interaction is seen at the center of the tetramer, and the distances are given in Table 3. In the G336V structure this hydrogen bond contact between opposite Asn<sup>190</sup> residues is also observed.

The G336V enzyme with bound serine crystallizes in two different space groups: form A is in P6<sub>2</sub>2<sub>2</sub> space group with one hydrogen bond contact between opposite Asn<sup>190</sup> residues is the distances are given in Table 3. In the G336V structure this hydrogen bond contact between opposite Asn<sup>190</sup> residues is also observed.

In crystal form A of G336V with bound serine (P6<sub>2</sub>,22), the subunit in the asymmetric unit forms the biological tetramer with its symmetry-related molecules. When this is superimposed on the G336V structure, there are domain movements in the range of 4.8–9 Å with the NBD and the regulatory domain-substrate binding domain acting as rigid bodies. This is in contrast to the native enzyme with serine bound where there are rotations of ~14° in subunits A and C and 4.5–6° in subunits B and D (Table 4). The intersubunit distance at Asn<sup>190</sup> between subunits A and C and subunits B and D are the same (5.9 Å), slightly less than that seen in the serine-bound native PGDH.

In contrast, in crystal form B of G336V with serine bound (P2<sub>1</sub>,2,2), there is no domain movement seen in subunits A and C, and only rotations of 6.0–6.5° are observed in subunits B and D. However, there is not much difference in the serine-bound mutant structures in these two different space groups as the r.m.s.d. of the monomer with subunit A is 0.56 Å and with subunit B is 0.81 Å for Cα atoms, and the overall r.m.s.d. of the tetramer is 1.15 Å for Cα atoms. The intersubunit distances of Asn<sup>190</sup> residues, 5.2 Å in subunits A and C and 4.8 Å between subunits B and D, are slightly less than in crystal form A and considerably less than seen in native PGDH with serine bound.

Numerous attempts were made to obtain good quality crystals of the double mutant G336V,G337V. Unfortunately they diffracted poorly and did not yield sufficient data. However, the double mutant structure with bound serine was solved at 2.5-Å resolution in space group P2<sub>1</sub>,2,2. In this case also there is a large difference in the unit cell parameters compared with the serine-bound native enzyme (~8 and 13 Å for a and b, respectively). Once again, rotation of the nucleotide binding domain relative to the rest of the molecule is not observed for subunits A and C, but there is a decrease in the intersubunit distance between Asn<sup>190</sup> residues (3 Å), which form a hydrogen bond very similar to that in the native enzyme. On the other hand, the rotation for subunits B and D appears to increase to ~10–12° with an increase in intersubunit distance between Asn<sup>190</sup> residues of 6–7 Å. A superposition of the G336V,G337V double mutant with serine bound onto the G336V mutant without serine is shown in Fig. 4. Because the structure of G336V is essentially identical to that of the native enzyme, this figure shows the domain rotation that occurs only in subunits B and D in the double mutant upon serine binding.

From the native structure, we know that upon serine binding the domain movements are a result of rotational changes.
TABLE 3
Distances between Asn\textsuperscript{190} on opposite subunits (Å)

| Subunits | Distances (Å) | R.m.s.d. (°) |
|----------|--------------|--------------|
| Native   | 3.63         | 5.8          |
| Native + serine | 8.1      | 3.17         |
| G336V    | 5.9          | 4.75         |
| G336V + serine (P6,22) | 5.21 | 3.22        |
| G336V,G337V + serine | 3.22 | 6.3        |

TABLE 4
Domain rotations

The relative domain rotation in degrees is defined in terms of the rotation of the NBD relative to the substrate binding domain and regulatory domain as a single unit.

| Serine subunit | Rotation |
|----------------|----------|
| Native vs. native + serine | A 2.04 14.5 |
| B 0.94 4.5 |
| C 1.78 13.9 |
| D 1.0 6.0 |
| G336V vs. G336V + serine (P6,22) | A 0.88 5.2 |
| B 0.73 4.9 |
| C 0.94 7.6 |
| D 1.28 9.0 |
| G336V vs. G336V + serine (P2,2,2) | A 0.75 0 |
| B 0.85 6.0 |
| C 1.0 0 |
| D 0.94 6.5 |
| G336V vs. G336V,G337V + serine | A 0.69 0 |
| B 1.57 11.8 |
| C 0.73 0 |
| D 1.26 10.0 |

Effect of Hinge Mutations on E. coli PGDH

FIGURE 3. Comparison of the experimentally determined inhibition of activity of G336V PGDH with that calculated with Equation 3. The experimentally determined inhibition profile (○) is compared with that calculated with Equation 3 (■) as a function of l-serine concentration.

at the two strands of peptide that link the NBD with the regulatory domain-substrate binding domain. This includes a \( \psi \) change of 181° for Ile\textsuperscript{293} (peptide flip), a \( \phi \) change of 150° for Gly\textsuperscript{294}, and a \( \psi \) change of 177° for Gly\textsuperscript{295}. In contrast to the native enzyme structure, these large changes in \( \phi \) and \( \psi \) angles are not observed in these mutant structures. Rather the domain rotations appear to come about as a result of smaller angle changes to a larger number of residues throughout the domain-linking polypeptides. For instance, in the connecting strand between Thr\textsuperscript{290} and Gln\textsuperscript{298}, \( \phi \) angle changes are not larger than 10°, and \( \psi \) angle changes are not larger than 27°. Similarly for the strand between Asn\textsuperscript{103} and Arg\textsuperscript{110}, \( \phi \) angle changes are not larger than 34°, and \( \psi \) angle changes are not larger than 45°.

As with the G336V structure, an inspection of the serine binding sites with serine bound does not reveal any obvious differences that might explain the reduction in affinity of these sites for serine in the mutant enzymes. When serine binds, Pro\textsuperscript{348} closes over the binding pocket shielding the serine from the solvent. The distance that the Pro\textsuperscript{348} \( C_\alpha \) atom moves upon serine binding is similar to that in the native enzyme and in all of the mutants studied.

One other area of note involves residues Ala\textsuperscript{344} and Gly\textsuperscript{145} that lie in a loop leading to Trp\textsuperscript{139} and Lys\textsuperscript{141} at the active site and that have been shown previously (39) to be critical for the cooperativity of serine inhibition. In the native enzyme, these residues undergo significant \( \phi \), \( \psi \) angle changes upon serine binding. Similar changes are also seen in these mutant structures with the exception of G336V crystal form B.

DISCUSSION

Previous studies indicated that the double glycine residues at positions 336 and 337, in the loop between the regulatory domain and the substrate binding domain, play a major role in the ability of l-serine to inhibit the activity of PGDH. This was demonstrated by the observation that replacing these residues with those of greater bulk reduced the sensitivity of the enzyme to l-serine. This was interpreted to be the result of a reduction in the rotational flexibility of the main chain bonds in this region. This was further supported by the demonstration that introducing new areas of flexibility (glycine residues) adjacent to these mutations could restore and even increase the sensitivity to serine over that of the native enzyme.

The serine binding data presented in this study demonstrates that the reduced sensitivity to serine resulting from mutations at Gly\textsuperscript{336} and Gly\textsuperscript{337} can be accounted for by a reduction in the affinity for serine rather than a block in the ability of the serine to affect the active site. That is, the quantitative relationship (Equation 3) that was determined for the native enzyme (9) between the ability of serine to bind to the effector site and the inhibition of activity at the active site also holds for the G336V mutant. Because the affinity of serine for the double mutant was too low to measure by equilibrium dialysis, we were not able to ascertain whether the degree of inhibition of this mutant can still be completely accounted for by Equation 3. Nonetheless both mutants, G336V and G336V,G337V, could be inhibited to greater than 95% by saturating amounts of serine.

The crystal structure of the G336V mutant described in this study clearly shows that it is very similar to the native enzyme with the only large and consistent change being in the apparent \( \phi \) and \( \psi \) angles at the point of the mutation. Angle changes are also seen at these positions as a result of serine binding to the
Effect of Hinge Mutations on E. coli PGDH

Figure 4. Superposition of G336V,G337V + serine with G336V tetramers. Left, the figure depicts a superposition of serine-bound G336V,G337V double mutant onto a G336V tetramer. The serine-bound G336V,G337V tetramer is generated using the symmetry-related molecules. Subunit A and C are colored orange, and subunits B and D are colored pink. In the G336V structure, the subunits A and C are colored with shades of green, and subunits B and D are colored with shades of blue. Subunits A and C of G336V,G337V + serine superimpose well on the respective A and C subunits of the G336V serine-free enzyme. In subunits B and D, there is rotation of the nucleotide binding domain of 10–12°. In both the tetramers, ligands are not shown for the purpose of clarity. Right, detailed view of the hydrogen bond interaction at Asn190 of subunits A and C of G336V,G337V + serine. In subunits B and D (pink), the Asn190 residues are separated by too great a distance to form hydrogen bond contacts.

native enzyme. Interestingly the mutants did not produce any significant changes in the serine binding site itself, and no obvious differences were noted at this site when comparing the native enzyme to the mutant enzymes after serine binds. Thus, it appears that the mutations at residues 336 and 337 are sufficient to reduce the affinity of the enzyme for serine, and whatever transitions are taking place in this region as a result of serine binding are hindered, but not eliminated, by the perturbations introduced by the valine mutants.

A very important observation resulting from the crystal structures of these mutants was that not all of the structural transitions seen when serine binds the native enzyme were observed when serine binds to the G336V and G336V,G337V mutants. Thus, those changes that are present in the mutant + serine structures appear to represent the minimal changes needed for inhibition of the active site. In this regard, several aspects of the mutant structures are remarkable. Among these is the absence of the previously reported large φ and ψ angle changes at the putative Gly294-Gly295 hinge region, suggesting that changes at these particular residues are not in themselves necessary for the inhibition of the enzyme.

The domain rotations of the NBD relative to the substrate binding domain-regulatory domain in the mutants are significantly different from the ~15° rotation originally reported for the native enzyme (7). The fact that the domain rotations observed in the mutant structures do not mimic those seen for the native enzyme upon serine binding suggests that they may not be necessary, at least to the degree originally reported, for inhibition of the active site. Rather they may represent a lower energy state in the native enzyme for accommodating the subunits within the tetramer in the presence of serine that is blocked by these mutations. Domain rotations of 5–6° in at least two subunits are seen consistently upon serine binding in all of the enzyme forms that have been studied. Thus, this may represent a critical conformational change in the inhibition of the active site by serine binding at the allosteric site.
Furthermore serine binding is consistently associated with breaking of the hydrogen bonds between two pairs of Asn residues found diagonally opposed at the tetrameric nucleotide binding domain interfaces. In the native enzyme + serine and both crystal forms of G336V + serine, the hydrogen bond is broken at both subunit pairs, A to C and B to D. However, in G336V,G337V + serine, the hydrogen bond is broken in only one subunit pair, B to D, suggesting that this may be sufficient for complete inhibition to occur consistent with a half-the-sites mechanism. In other words, both Asn hydrogen bonds are necessary for the enzyme to be active.

Previous studies have shown that the half-of-the-sites activity of the enzyme corresponds closely to the flip-flop mechanism first discussed by Lazdunski (40) where the active subunits alternate from one pair to the other. Although additional investigation needs to be done in this regard, it is interesting to consider if the intersubunit hydrogen bonds between Asn residues may somehow have a role in linking the active subunit pairs. This could potentially explain why the breaking of only one of the pairs, as seen in the double mutant structure, is sufficient to cause essentially complete inhibition.

It has also been shown (39) that \( \phi \) and \( \psi \) angle changes at residues Ala\(^{144} \) and Gly\(^{145} \) may be critical especially in the cooperativity of inhibition. When serine binds to the effector site in the regulatory domain of PGDH, the activity of the enzyme is inhibited in a positively cooperative manner with a Hill coefficient of \( \sim 2 \). This is an interesting and potentially important region as Ala\(^{144} \) and Gly\(^{145} \) lie in a bend of a loop leading to Trp\(^{139} \) and Lys\(^{141} \). Trp\(^{139} \) inserts into a pocket at the base of the active site in the adjacent subunit, and conversion of Trp\(^{139} \) to glycine results in a greatly reduced activity and a decrease in the Hill coefficient to near 1 (38). Lys\(^{141} \) contributes a positive charge to the active site of the adjacent subunit. Conversion of Lys\(^{141} \) to alanine produces an enzyme with homotropic cooperativity with respect to substrate (39). Thus, this loop provides a potential route connecting serine binding to the active site across adjacent subunits. Conversion of Gly\(^{145} \) to valine exhibits a 23-fold decrease in the \( k_{cat}/K_m \) and a decrease in the Hill coefficient to 1.2. Conversion of Ala\(^{144} \) to valine produces a 2-fold increase in the \( k_{cat}/K_m \) and also reduces the Hill coefficient to 1.2 (39). The mutagenesis data strongly suggest the involvement of these residues in the modulation of the active site by serine. Significant \( \phi, \psi \) angle changes are observed at these residues when serine binds in all of the structures except in crystal form B (P2\(_1\),2\(_1\),2) of the G336V mutant + serine. These include the serine-bound forms of the native enzyme, crystal form A (P6\(_2\),22) of the G336V mutant, and the G336V,G337V mutant. In the native enzyme and crystal form A of G336V, these changes are observed in all subunits, whereas in G336V,G337V they are observed only in subunits B and D. In the latter, these are the same two subunits that display a 10 –12° rotation of the nucleotide binding domain and the lack of hydrogen bonding between Asn residues. The supporting data favor the involvement of Ala\(^{144} \) and Gly\(^{145} \) in the cooperative process. However, the apparent inconsistency in the observed angle differences upon serine binding in the G336V crystal form A leaves the question of whether or not they serve as an additional hinge region unresolved.

This investigation demonstrated that the effect of mutation at the Gly\(^{336} \) and Gly\(^{337} \) positions is severalfold. There is a direct effect on the affinity of the serine binding sites for serine without producing any observable changes in the structures of the sites themselves. Therefore, the Gly\(^{336} \),Gly\(^{337} \) “hinge” region appears to function mainly in the transition that occurs upon serine binding. In addition, inhibition occurs in the absence of many of the large scale domain rotations first observed when the structure of the native enzyme was compared with the native enzyme with bound serine. This observation is critical in defining the minimal conformational changes that occur upon serine binding and that lead to inhibition of the active site. In conjunction with the domain rotations, these studies further established the role of both of the intersubunit Asn\(^{190} \) hydrogen bonds as being critical to the active enzyme. Overall this provides valuable information in the continuing investigation of the mechanism of allosteric regulation in PGDH and the function of the small molecule binding ACT domain as a regulatory element.

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. Grant, G. A., Scheller, D. J., and Banaszak, L. J. (1996) Protein Sci. 5, 34–41
4. Al-Rabiee, R., Zhang, Y., and Grant, G. A. (1996) J. Biol. Chem. 271, 23235–23238
5. Grant, G. A., Xu, X. L., and Hu, Z. (1999) Protein Sci. 8, 2501–2505
6. Scheller, D., Grant, G. A., and Banaszak, L. J. (1995) Nat. Struct. Biol. 2, 69–76
7. Thompson, J. J., Bell, J. K., Pratt, J., Grant, G. A., and Banaszak, L. J. (2005) Biochemistry 44, 5763–5773
8. Grant, G. A. (2006) J. Biol. Chem. 281, 33825–33829
9. Grant, G. A., Xu, X. L., and Hu, Z. (2004) J. Biol. Chem. 279, 13452–13460
10. Grant, G. A., Xu, X. L., and Hu, Z. (2000) Biochemistry 39, 7316–7319
11. Al-Rabiee, R., Lee, E. J., and Grant, G. A. (1996) J. Biol. Chem. 271, 13003–13017
12. Scheller, D. J., Fetter, C. J., Banaszak, L. J., and Grant, G. A. (1989) J. Biol. Chem. 264, 2645–2648
13. Tobey, K. L., and Grant, G. A. (1986) J. Biol. Chem. 261, 12179–12183
14. Zhao, G., and Winkler, M. E. (1996) J. Bacteriology 178, 232–239
15. Grant, G. A., and Xu, X. L. (1998) J. Biol. Chem. 273, 23389–23394
16. Bell, J. E., and Bell, E. T. (1988) Proteins and Enzymes, pp. 465–470, Prentice-Hall, Inc., Englewood Cliffs, NJ
17. Grant, G. A., Hu, Z., and Xu, X. L. (2001) J. Biol. Chem. 276, 17844–17850
18. Grant, G. A. (2004) Methods Enzymol. 380, 106–131
19. Philo, J. S., Talvenheimo, J., Wen, J., Rosenfeld, R., Welcher, A. A., and Arakawa, T. (1994) J. Biol. Chem. 269, 27840–27846
20. Philo, J. S. (2000) Methods Enzymol. 312, 100–120
21. Laut, T. M., Shah, B. D., Ridgway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Har- ding, S. E., Rowe, A. J., and Horton, J. C., eds) pp. 90–125, Royal Society of Chemistry, Cambridge, UK
22. Schuck, P. (2000) Biophys. J. 78, 1606–1619
23. Philo, J. S. (1994) in Modern Analytical Ultracentrifugation (Schuster, T. M., and Laue, T. M., eds) pp. 156–170, Academic Press, New York
24. Storoni, L. C., McCoy, A. J., and Read, R. J. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 432–438
25. Sauter, N. K., Grosse-Kunstleve, R. W., and Adams, P. D. (2004) J. Appl. Crystallogr. 37, 399–409
26. Leslie, A. G. W. (1992) J. CCP4/ESF-EAMCB Newsletter. Protein Crystallogr. 26
Effect of Hinge Mutations on E. coli PGDH

29. Evans, P. R. (1993) in Proceedings of CCP4 Study Weekend. Data Collection and Processing (Sawyer, L., Isaacs, N., and Bailey, S., eds) pp. 114–122, Daresbury Laboratory, Warrington, UK
30. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
31. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
32. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
33. Kantardjieff, K. A., Hochtl, P., Segelke, B. W., Tao, F. M., and Rupp, B. (2002) Acta Crystallogr. Sect. D Biol. Crystallogr. 58, 735–743
34. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
35. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
36. Hayward, S., and Berendsen, H. J. C. (1998) Proteins 30, 144–154
37. Rosenbloom, J., Sugimoto, E., and Pizer, L. I. (1968) J. Biol. Chem. 243, 2099–2107
38. Grant, G. A., Xu, X. L., and Hu, Z. (2000) Arch. Biochem. Biophys. 375, 171–174
39. Grant, G. A., Hu, Z., and Xu, X. L. (2005) Biochemistry 44, 16844–16852
40. Lazdunski, M. (1972) Curr. Top. Cell. Regul. 6, 267–310
41. Grant, G. A., Hu, Z., and Xu, X. L. (2003) J. Biol. Chem. 278, 18170–18176