Amino Acid Residue Mutations Uncouple Cooperative Effects in *Escherichia coli* d-3-Phosphoglycerate Dehydrogenase*

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**d-3-Phosphoglycerate dehydrogenase from *Escherichia coli*** contains two Gly-Gly sequences that occur at junctions between domains. A previous study (Grant, G. A., Xu, X. L., and Hu, Z. (2000) *Biochemistry* 39, 7316–7319) determined that the Gly-Gly sequence at the junction between the regulatory and substrate binding domain functions as a hinge between the domains. Mutations in this area significantly decrease the ability of serine to inhibit activity but have little effect on the $K_m$ and $k_{cat}$. Conversely, the present study shows that mutations to the Gly-Gly sequence at the junction of the substrate and nucleotide binding domains, which form the active site cleft, have a significant effect on the $k_{cat}$ of the enzyme without substantially altering the enzyme’s sensitivity to serine. In addition, mutation of Gly-294, but not Gly-295, has a profound effect on the cooperativity of serine inhibition. Interestingly, even though cooperativity of inhibition can be reduced significantly, there is little apparent effect on the cooperativity of serine binding itself. An additional mutant, G336V,G337V, also reduces the cooperativity of inhibition, but in this case serine binding also is reduced to the point at which it cannot be measured by equilibrium dialysis. The double mutant G294V,G336V demonstrates that strain imposed by mutation at one hinge can be relieved partially by mutation at the other hinge, demonstrating linkage between the two hinge regions. These data show that the two cooperative processes, serine binding and catalytic inhibition, can be uncoupled. Consideration of the allowable torsional angles for the side chains introduced by the mutations yields a range of values for these angles that the glycine residues likely occupy in the native enzyme. A comparison of these values with the torsional angles found for the inhibited enzyme from crystal coordinates provides potential beginning and ending orientations for the transition from active to inhibited enzyme, which will allow modeling of the dynamics of domain movement.

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1 The abbreviation used is: PGDH, d-3-phosphoglycerate dehydrogenase.

**d-3-Phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95)**

from *Escherichia coli* is a tetramer of identical subunits (1), the enzymatic activity of which is regulated in an allosteric manner by l-serine, the end product of its metabolic pathway (2–4). Each subunit contains three distinct domains that can be visualized easily in the crystal structure (1). These are referred to as the regulatory (or serine binding), substrate binding, and nucleotide binding domains (Fig. 1).

The binding sites for the substrate and the effector are formed by amino acid residues at the interfaces between domains (1). In both cases, hydrogen bonds are formed between the ligands and both domains forming the interface. Serine binds at the two interfaces formed by adjacent regulatory domains, forming intersubunit hydrogen bonds at these sites. The four active sites are formed by the juncture of the substrate binding domain and the nucleotide binding domain. The substrate interacts with adjacent domains within a subunit but also with residues in the adjacent subunit. A consequence of this configuration is that a single serine molecule makes contact with two subunits, each having an active site, and potentially each is affected directly by the single serine. Also as a consequence of this configuration, two separate cooperative effects are possible and are seen in the native enzyme. These are cooperativity in serine binding itself (5) and cooperativity of inhibition as a result of serine binding (2, 3, 6).

The regulatory domain is linked to the substrate binding domain by a single strand of polypeptide that contains a Gly-Gly sequence approximately midway between the two domains. The function of this Gly-Gly sequence has been investigated by site-directed mutagenesis (7), and the data are consistent with its functioning as a hinge region between the two domains. Mutations at this hinge affect sensitivity to serine without appreciably affecting catalytic activity (see Table 1).

In a similar manner, the substrate binding domain is linked to the nucleotide binding domain by two strands of polypeptide, one of which contains the only other Gly-Gly sequence in the enzyme. This linkage also comprises the locus at which the substrate binding domain and the nucleotide binding domain join to form the active site cleft. Amino acid residues within the active site cleft that seem to play a role in substrate binding and potentially in the regulation of the enzymatic activity have been identified previously (8). In order for the active site to close around the substrate during catalysis, it would seem that some flexibility is required in the vicinity of this Gly-Gly sequence. This study explores the role of this area as a potential hinge region for the functioning of PGDH and reveals its role in the cooperativity of serine inhibition.

**MATERIALS AND METHODS**

Mutants of PGDH were produced, expressed in *E. coli*, and isolated as described previously (6, 9). Catalytic activity was determined at 25° in 20 mM Tris buffer, pH 7.5, using a-ketoglutarate as the substrate, and by monitoring the decrease in absorbance of NADH at 340 nm (10, 11). Protein concentration was determined by the Bradford method as described previously (12, 13) and by quantitative amino acid analysis for the binding studies. All mutations are constructed in PGDH<sub>4C/A</sub> a...
form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine (12). The IC50 value for l-serine is the concentration of serine that produces a 50% inhibition of the enzyme activity. Kinetic parameters were determined either using direct linear plots (14) or by fitting the data to the Michaelis-Menten equation with Kaleidograph (Synergy Software). Enzyme homogeneity was judged by SDS gels. Figs. 2 and 3 were produced with MOLSCRIPT (15).

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 (8, 12) and a loss of serine binding (16). All mutants in this study maintained an emission maximum at 340 nm and retained their ability to bind serine, indicating an intact association of subunits.

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 was 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 $Y = [L]^{n}/[K + [L]^{n}]$ (17), where $Y$ is the fractional inhibition, $[L]$ is the concentration of ligand, $n$ is the Hill coefficient, and $K$ is the Hill constant. Serine binding data were fit to the Adair equation (17) for either four sites or two sites.

$$Y = \frac{4L/K_{1} + 2L^{2}/K_{2} + 3L^{3}/K_{3} + 4L^{4}/K_{4}}{4(1 + L/K_{1}) + (L/K_{2}) + (L^{2}/K_{3}) + (L^{3}/K_{4})}$$

or two sites

$$Y = \frac{(L/K_{1}) + (2L^{2}/K_{2})}{2(1 + L/K_{1} + L^{2}/K_{2})}$$

where $Y$ is the fractional occupancy, $L$ is the free ligand concentration, and $K_{i}$ are the stepwise Adair constants. Data were fit also to the equation for equivalent independent binding sites, $r = \rho[L]/[K_{d} + [L]]$, where $r$ is moles of ligand bound/mole of acceptor, $[L]$ is the free ligand concentration, and $\rho$ is the number of binding sites. Fitting was performed using Kaleidograph (Synergy Software). Intrinsic site dissociation constants were calculated from the Adair constants using the following statistical relationships for a molecule, where $n$ sites are occupied at maximal binding, and $K'_{i}$ values are the intrinsic dissociation constants (17).

$$k_{cat}/K_{M} = \frac{k_{cat}}{[S]_{0}} = \frac{1}{K_{M}}$$

$$n = 2 \text{ sites; } K'_{i} = 2K_{i}, K'_{s} = K_{s}/2$$

$$n = 3 \text{ sites; } K'_{i} = 3K_{i}, K'_{s} = K_{s}/3$$

$$n = 4 \text{ sites; } K'_{i} = 4K_{i}, K'_{s} = 3K_{s}/2, K'_{s} = 2K_{s}/3, K'_{s} = K_{s}/4$$

Thermodynamic linkage analysis using double mutant cycles was performed as described by Ackers and Smith (18) and by Horovitz and Fersht (19). In the mutant cycle shown in Equation 6, $N$ denotes a residue position that is not mutated and $M$ denotes a residue position that is mutated.

$$\Delta G_{1} \rightarrow \Delta G_{1}$$

$$\Delta G_{1} \rightarrow \Delta G_{12}$$

$$\Delta G_{12}$$

The change in $\Delta G$ is calculated with the equation

$$\Delta G = RT \ln(K_{M}/K_{s})$$

where the subscripts $m$ and $n$ indicate the value after and before mutation, respectively. $R$ is the universal Gas constant, and $T$ is the temperature in degrees Kelvin. The coupling or interaction energy, $\Delta G_{INT}$, is defined in terms of the respective $\Delta G$s as follows.

$$\Delta G_{INT} = \Delta G_{12} - \Delta G_{1} - \Delta G_{2}$$

If the two positions do not interact, the differences in $\Delta G$ will be non-zero. On the other hand, if they do interact, the differences in $\Delta G$ will have a non-zero value. In this study, interaction with respect to the active site is analyzed using the overall rate constant $k_{cat}/K_{M}$ (18), and interaction with respect to the serine binding site is analyzed using the IC50 value as a global approximation of the overall binding of serine.

**RESULTS**

Fig. 2 shows the location of the Gly-Gly sequence at the active site of PGDH in relation to His-292 and NADH, which participate in the proton and hydride transfer during catalysis. The active site cleft lies to the left of the arc made by the polypeptide chain. There do not seem to be any hydrogen bond-
ing contacts made between this segment of polypeptide with other segments of the enzyme. Note that the structure shown is derived from the inhibited enzyme so that hydrogen bonding in this area may be different in the active enzyme.

Fig. 3 shows an α-carbon tracing of a subunit of PGDH with the approximate location of the two Gly-Gly sequences noted above. Gly-294 and Gly-295 lie at one end of an α-helix extending to Gly-336 and Gly-337. Thus, this helix is a potential route for the transmission of conformational information from the regulatory domain to the active site. A previous report (7) explored the role of Gly-336 and Gly-337 by producing mutants with side chains of increasing bulk at these positions. The effect of removing bulky side chains from flanking positions was investigated also. A similar strategy is used in these studies.

The kinetic results (Table I) show that the ability of serine to inhibit catalytic activity varies by no more than 2-fold in mutants not involving Gly-336 or Gly-337. On the other hand, $k_{cat}/K_m$ values are affected by as much as three orders of magnitude with the largest effect seen with respect to $k_{cat}$. Replacement of the glycine residues at either position 294 or 295 with alanine only has a minimal effect. A major effect on the kinetic parameters is not evident until either glycine residue is replaced with a valine side chain. In both cases, the effect is primarily on the $k_{cat}$ of the enzyme. Shifting the Gly-Gly sequence one position to the left, while maintaining a valine at position 295 (I293G/G295V), seems to result in an approximately one order of magnitude increase in $k_{cat}/K_m$ compared with the mutants containing only a single glycine residue. A similar shift to the right (G294V/S296G) has little effect.

Interestingly, an inspection of the Hill coefficients shows that mutation of Gly-294 has a substantial effect on the cooperativity of the inhibition by L-serine. In all cases where the addition of a side chain at position 294 produces a significant effect on the $k_{cat}$, it also decreases the Hill coefficient of the inhibition curve from ~2 to values close to 1 (G294V, G294V/S296G, and G294V/G295V). A similar effect is not evident when comparable mutations are performed at position 295 (G295V and I293G/G295V). Both G294V and G295V mutants

**Fig. 3. α-carbon chain tracing of a subunit of PGDH depicting the location of the two Gly-Gly sequences.** The Gly-Gly sequence that is the subject of this study (Gly-294–Gly-295) is the lower one. It is depicted in Fig. 1 in more detail. The α-helix that extends from the vicinity of one Gly-Gly sequence to the other is shaded black.

**Fig. 4. Inhibition curves for G294V and G295V.** Top, inhibition of G294V (○) compared with unmutated (4C/A) enzyme (■). Bottom, inhibition of G295V (○) compared with unmutated (4C/A) enzyme (■).
sic dissociation constants indicate an element of positive coop-


term for four-site Adair equation is shown in Fig. 5. The derived intrin-


ces yield similar results. The graphical analysis for the


ting procedures are presented in Table II along with the


tion for either two or four sites and to the equation for equiv-


tion with the Gly-294 mutant was tested by analyzing the


tution with the Gly-294 mutant was tested by analyzing the


tion of the enzyme and remains in question. This binding behavior


ty is sensitive to the quantitation


t occupancy of a third site. However, the appearance of partial


t data show that a maximum


t was performed to look at the effect of mutating Gly-294 on


tive for inhibition has been reduced to near 1 (1.13).


t coefficient. Representative inhibition curves, illustrating


t double mutant G294V,G336V. Interestingly, the value of


t determination with the Gly-294 mutant was tested by analyzing the


t to the substrate binding domain indicated that


t least two sites are occupied fully with the possibility of partial


t of the native en-


t behavior is very similar to that reported previously for the native en-


t of two sites are occupied fully with the possibility of partial


t of two or four sites are occupied fully with the possibility of partial


t behavior is very similar to that reported previously for the native en-


t of the enzyme and remains in question. This binding behavior


t sensitivity to serine without affecting the kinetic parameters or


t to bind ligand.


t of sites, respectively, for two sites as fractional occupancy of sites (Y) versus


t the data to the Adair equation for a molecule with four sites. The bottom panel shows the distribution of residuals for the fit.


t significantly reduce $k_{cat}/K_m$ values, but only G294V reduces the


tes the loss in sigmoidicity for G294V, are shown in Fig. 4.


t direct analysis of L-serine binding by equilibrium dialysis


t data to the Adair equation for a


t for two or four sites. The


t for equivalent independent sites. In addition, the residuals for


t for the Adair equation, which allows for the analysis of depend-


t the Hill coefficient. Representative inhibition curves, illustrating


tic dissociation constants indicate an element of positive coop-


t the data to the Adair equation for a


t to the Adair equation indicates a significantly better fit than the values of 2.2 and 1.5 for the fits for equivalent independent sites. In addition, the residuals for


t for the fit to the Adair equation are much smaller and more consist-


tic dissociation constants derived from these fitting procedures are presented in Table II along with the


tic dissociation constants indicate an element of positive coop-


t observed for the G294V,G336V double mutant. The


t shift in the dissociation constant for the second site


t sensitivity to serine without affecting the kinetic parameters or


t in conjunction with the Gly-294 mutant was tested by analyzing the
ticate for inhibition has been reduced to near 1 (1.13).


t reported previously, the serine sensitivity of G336V,


t netic and inhibition parameters


| Mutant       | Residue number | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ | IC$_{50}$ Ser | Hill coefficient |
|--------------|----------------|-------|-----------|-----------|---------------|----------------|------------------|
|              | 292 294 295 296 336 337 |       |           |           |               |               |                  |
| 4C/A         | I G G S G G G | 0.6 ± 0.14 | 28.9 ± 3.9 29.0 | 4.8 × 10$^4$ | 10 | 2.02 ± 0.07 |                  |
| G294A        | I A G S G G G | 0.85 ± 0.09 | 23.4 ± 0.9 10.8 | 1.3 × 10$^4$ | 8 | 1.82 ± 0.04 |                  |
| G294V        | I V G S G G G | 0.77 ± 0.07 | 19.0 ± 0.6 0.079 | 1.0 × 10$^2$ | 15 | 1.08 ± 0.04 |                  |
| G295A        | I G A S G G G | 1.2 ± 0.06 | 43.6 ± 1.0 23.4 | 2.0 × 10$^4$ | 8 | 2.15 ± 0.13 |                  |
| G296V        | I G V S G G G | 0.58 ± 0.04 | 7.2 ± 0.2 | 0.021 | 3.6 × 10$^4$ | 15 | 2.11 ± 0.20 |                  |
| G294V,G295V  | | 18.8 ± 1.2 | 0.022 | 2.4 × 10$^4$ | 23 | 1.26 ± 0.24 |                  |
| I293G,G295V  | G V G S G G G | 0.49 ± 0.004 | 18.8 ± 0.05 | 0.24 | 4.9 × 10$^2$ | 9 | 1.87 ± 0.09 |                  |
| G294V,S296G  | I V G G G G G | 0.48 ± 0.02 | 14.2 ± 0.2 | 0.09 | 1.9 × 10$^2$ | 20 | 1.11 ± 0.07 |                  |
| G294V,G336V  | I V G S V V G | 0.08 ± 0.01 | 5.8 ± 0.09 | 0.048 | 6.0 × 10$^2$ | 75 | 1.36 ± 0.07 |                  |
| G336V$^{IV}$ | I G G S V G V | 0.25 ± 0.05 | 21.1 ± 0.7 | 19.0 | 7.6 × 10$^4$ | 142 | 1.82 ± 0.09 |                  |
| G337V$^{IV}$ | I G G S V G V | 0.15 ± 0.02 | 29.6 ± 0.7 | 6.9 | 4.6 × 10$^2$ | 15 | 2.24 ± 0.14 |                  |
| G336V,G337V$^{IV}$ | | 0.10 ± 0.02 | 21.5 ± 0.7 | 9.0 | 9.0 × 10$^4$ | 800 | 1.20 ± 0.05 |                  |

* Originally reported in Ref. 7.

G337V is decreased substantially, but there is essentially no


t mutations is on the ability of the enzyme to bind serine. The kinetic parame-


ts K$_m$, V$_{max}$, and the Hill coefficient are comparable with that of 213, 4, and 56 μM determined


tability domain to the substrate binding domain indicated that


t only Gly-336 exhibited an effect when bulky side chains were


t study (7) of the Gly-Gly pair linking the regulat-


t to bind ligand.


t previous study (7) of the Gly-Gly pair linking the regu-


t at this position. In this case, G336V (Table I) decreased


t two sites as fractional occupancy of sites (Y) versus


t effect of substituting Gly-294 for G294V, but significant positive cooperativity still exists with an almost


t dissociation constant for the second site to bind ligand.


to the Adair equation with the Gly-294 mutant was tested by analyzing the double mutant G294V,G336V. Interestingly, the value of


t double mutant G294V, G336V. Serine binding analysis of the G294V,G336V mutant shows very clearly the sigmoidal nature of the binding curve (Fig. 6) even though the Hill coefficient for inhibition has been reduced to near 1 (1.13).

As reported previously, the serine sensitivity of G336V,
Uncoupling of Cooperativity in PGDH

TABLE II
Analysis of binding data for G294V

| Mutants      | kcat/Km (1) | IC50 (μM) |
|--------------|-------------|-----------|
| G294V,G295V  | -3.4        | 0         |
| G294V,G336V  | -1.2        | 1.0       |
| G336V,G337V  | -0.1        | -0.8      |

DISCUSSION

This paper is the second in the investigation of the role of the Gly-Gly sequences found at the junctures of the domains of PGDH. The previous study (7) showed that in the case of the mutation of Gly-336 and Gly-337, the value for kcat/Km remained near that of the native enzyme for all mutations studied. However, the ability of serine to inhibit the enzyme was decreased by as much as 350-fold (7) for some mutants. For mutation of Gly-294 and Gly-295, the opposite effect is observed (Table I). That is, large differences in kcat are seen without appreciable effects on the enzymes sensitivity to serine. These observations are consistent with the hypothesis that the link between the regulatory and substrate binding domains is functional in linking serine binding to active site inhibition, whereas the link between the substrate and nucleotide binding domains is functional in the dynamics of catalysis.

In the previous study, the decrease in serine sensitivity produced by adding a bulky side chain to position 336, or both 336 and 337, could be relieved completely by placing a glycine at the flanking position 335 (7). Thus, the new glycine seemed to relieve the effect of the mutation by providing a new hinge point in the connecting segment. A similar reversal of mutational effect, but to a less significant degree (only ~10×), is seen by placing a glycine residue at position 293 when position 295 is a valine. This suggests that if rotational freedom is critical at this locus, it is accommodated more narrowly. However, this remains a likely interpretation because no obvious steric interference of the mutant residue with a neighboring structure is evident from the crystal structure.

The data also indicate clearly that Gly-294 is critical for the cooperativity of L-serine inhibition, an observation that is seen consistently throughout the mutants studied. Whenever a bulky side chain is placed at this position, the Hill coefficient is decreased substantially to a value near 1. This appears to be a function of the bulk of the side chain rather than just the absence of glycine because the effect is not seen when Gly-294 is converted to an alanine.

Interestingly, although a mutation at Gly-294 reduces cooperativity in serine inhibition, it seems to have only a minimal effect on the cooperativity of serine binding to the enzyme. The best fit to the data for G294V is produced by the Adair equation and indicates that the binding sites for serine are not independent. Although the sigmoidal shape of the binding curve for G294V is not pronounced, it is evident. This conclusion is substantiated by the observation that the sigmoidal nature of the binding curve for the G294V,G336V mutant is especially pronounced, and it also produces a Hill coefficient for binding near 1.

For G294V, an apparent reduction in the first intrinsic dissociation constant is seen relative to the unmutated form, but the general characteristics of substantial positive cooperativity in the binding of the second ligand and general lack of binding to the fourth and perhaps the third site are still apparent. For G294V,G336V, the first intrinsic dissociation constant is on the order of 500 μM, whereas the second is ~8 μM, again reflecting significant positive cooperativity. Hence, placing a bulky side...
chain at position 294 seems to uncouple the two cooperative processes effectively.

Classically, allosteric proteins have been viewed globally as being in either one state or another. Thus, one might assume that when two processes are affected by the same stimulus (serine binding) they both should be affected equally. This may be true for native enzyme, but when mutations are introduced at specific structural loci, the potential for splitting transmission to distant points on the structure exists. Thus, such uncoupling of cooperative effects should be possible theoretically if the end point for each is not along the same conformational pathway. In this case, serine binding directly affects two separate structural loci on the tetrameric protein. These are the other serine binding sites located at the two regulatory domain interfaces on the one hand and the catalytic sites located between the substrate binding domain and nucleotide binding domain on the other. Because these functionally different sites are located spatially apart on the protein, the pathway of conformational transduction from the first serine binding event to each of them must diverge structurally at some point. If a mutation such as G294V is located past the point of divergence but before one of these loci (i.e. the catalytic site), it seems reasonable that the mutant could affect cooperativity at that site to a much greater extent than at the other site, the transduction pathway of which has not been altered. The data presented here suggest that this is the case.

A similar effect on cooperativity is not seen with a mutation at Gly-295. Although a valine at position 295 decreases the $k_{cat}$ even more than Gly-294 (by an additional 4-fold), no change in the cooperativity of inhibition occurs. A reason for this lack of change is not suggested immediately by the inhibited structure, although it is noted that Gly-294 is spatially between Gly-295 and the His-292/Pro-291/Trp-139 cluster (see Fig. 2), which has been suggested (8) as a possible locus for direct interaction of the active site residues with the adjacent subunit. Trp-139 from the adjacent subunit inserts into a hydrophobic pocket at the base of His-292, the catalytic histidine. Mutation of Trp-139 to smaller side chains also has a substantial effect on the cooperativity of L-serine inhibition. Gly-294 is only two residues removed from His-292 and three residues from Pro-291, which forms part of the Trp-139 pocket and is only $\sim$3.6 Å from the indole face of the tryptophan side chain in the adjacent subunit. Thus, Gly-294 is in an ideal position to affect not only the position of a critical catalytic residue but also the direct interaction between residues from two adjacent subunits. This is consistent with both the decrease in catalytic efficiency as well as the decrease in cooperativity that is seen experimentally.

Although the mutant G294V,G336V also produces a Hill coefficient near 1, the values for $k_{cat}/K_m$ and serine inhibition (IC$_{50}$) are intermediate between those for G294V and G336V. This indicates that a mutation in one area can affect the other area, as born out in the linkage analysis shown in Table III. The opposite signs of the $\Delta G_{int}$ for kinetic and serine binding parameters indicate a favorable outcome for both functionalities, thus confirming that the effects can travel in both directions. That is, a mutation at the catalytic site can relieve strain at the serine binding sites caused by a mutation in that region and vice versa.

Because the addition of side chains at the glycine positions would have the effect of restricting rotation about the bonds described by the $\Phi$ and $\Psi$ angles of these residues, these two pairs of glycine residues seem to function as rotational hinges in the dynamics of catalysis and inhibition. The $\Phi$ and $\Psi$ angles for the glycine residues in the two Gly-Gly pairs are shown in the Ramachandran plots in Fig. 7. Keep in mind that these angles are for the inhibited enzyme; the angles found in the native enzyme are not known. However, the mutation data can be used to give an indication of what the ranges may be for the active enzyme. If a mutation has no effect on a particular function, then it can be assumed that the $\Phi$ and $\Psi$ angles of the active enzyme are likely within the areas allowed for the side chain placed at that position. If a mutation negatively affects a particular function, then the assumption is that the $\Phi$ and $\Psi$ angles of the fully active enzyme are not within the allowed regions for that side chain. One must keep in mind that these allowed regions are only approximate because the allowed Ramachandran angles were determined with rigid-sphere models, and there are many examples of structures in which residues apparently occupy forbidden space.

The $\Phi$ and $\Psi$ angles for Gly-294, -295, -336, and -337 in the inhibited enzyme place all these residues in or very close to (Gly-294) the allowed regions for glycine residues. However, Gly-294 and Gly-336 are outside the allowed areas for alanine and valine. These are also the two residues that when mutated produce the most profound effects on serine sensitivity and the cooperativity of serine inhibition.

When Gly-294 or Gly-295 are converted to alanine there

![Fig. 7. Ramachandran plots showing the torsional angles for Gly-294, -295, -336, and -337 in the inhibited enzyme. The generally allowed areas for glycine, alanine, and valine residues are shown also (22, 23). The figure depicts two Ramachandran plots side by side. Left, the lightly shaded areas show the generally allowed areas for glycine, and the darker shaded areas show the generally allowed areas for valine. Note that a rotation of $+180^\circ$ is equivalent to a rotation of $-180^\circ$. Therefore, the rotational space depicted at the junction of the two plots is contiguous. The figure is drawn in this manner to highlight the proximity of the angles for Gly-294 and Gly-336 to the allowed alanine and valine areas.](image)
essentially is no change in activity or serine binding, but conversion to valine significantly reduces $k_{cat}/K_m$ for both. Therefore, in the active enzyme both of these residues likely have $\Phi$ and $\Psi$ angles within the region allowable for alanine. When serine inhibits the enzyme, the $\Phi$ and $\Psi$ angles for Gly-295 are still within this region, but those for Gly-294 are well outside this region, suggesting that serine binding causes a significant rotation at this residue.

When Gly-336 is converted to alanine or valine (Ref. 7 and Table I) the IC$_{50}$ values for serine increase ~4- and 14-fold, respectively. Therefore, the values for the active enzyme for position 336 are likely outside of the areas allowed for alanine and valine because they are in the inhibited enzyme. There is no change in IC$_{50}$ for the mutation of Gly-337, and so the $\Phi$ and $\Psi$ angles for this position in the active enzyme are likely also to be within the areas allowed for valine. In the inhibited enzyme, Gly-337 lies within the area allowed for alanine but not for valine, suggesting a small shift during inhibition.

Based on these data, it is possible to deduce a likely range of values for the $\Phi$ and $\Psi$ angles at these hinge positions in the active enzyme, and by comparing that to the $\Phi$ and $\Psi$ angles observed in the inhibited enzyme, we can start to address what happens to the relative positions of the subunit domains during this transition. Modeling this transition will not be trivial, however, and is well outside the scope of this study. Potential compensation by rotations of other residues will have to be taken into account as well as accommodating any domain motion within the context of maintaining the tetramer contacts.

The role of Gly-294 and that of the combination of Gly-336 and Gly-337 in the cooperativity of serine inhibition are particularly intriguing. We have shown previously (21) that mutations at the interface between the regulatory and substrate binding domains can decrease the cooperativity of serine inhibition while at the same time increasing the sensitivity of the enzyme to being inhibited by serine. This suggests that each active site is affected by serine binding to its own subunit in a noncooperative manner and that additional interaction between domains propagates the cooperative effect. When Gly-294 is converted to valine, it seems serine binding can affect its torsional angles to the extent of producing inhibition, but the torsional angles are not changed to the extent that they will propagate the cooperativity. When both Gly-336 and Gly-337 are converted to valine, not only is serine binding significantly decreased, but so is the cooperativity of remaining inhibition.

This is similar to the effect seen when the regulatory/substrate binding domain interface residues themselves are altered (21). Thus, the common effect of these two different mutants, reducing cooperativity of inhibition, seems to come about for two different reasons. G336V,G337V most likely is not able to produce the domain interface contacts to sufficiently alter the torsional angles of Gly-294 required for cooperativity between subunits. On the other hand, G294V is most likely sterically restrained from achieving the necessary torsional angle transition for the cooperative effect.

The observation that cooperativity of inhibition can be uncoupled from cooperativity of effector binding underscores the concept that the two processes occur by distinct pathways initiated by a common event. Furthermore, there is clearly linkage between these two areas. The degree to which the two pathways share common features and the nature of their linkage in the native enzyme can now begin to be addressed.

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