Role of an Interdomain Gly-Gly Sequence at the Regulatory−Substrate Domain Interface in the Regulation of *Escherichia coli*. D-3-Phosphoglycerate Dehydrogenase†

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ABSTRACT: The regulatory and substrate binding domains of D-3-phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95) from *Escherichia coli* are connected by a single polypeptide strand that contains a Gly-Gly sequence approximately midway between the domains. The potential flexibility of this sequence and its strategic location between major domain structures suggests that it may function in the conformational change leading from effector binding to inhibition of the active site. Site-directed mutagenesis of this region (Gly-336−Gly-337) supports this hypothesis. When bulky side chains were substituted for the glycines at these positions, substantial changes in the ability of serine to inhibit the enzyme were seen with little effect on the activity of the enzyme. The effect of these substitutions could be alleviated by placing a new glycine residue at position 335, immediately flanking the original glycine pair. On the other hand, substituting a glycine at position 338 revealed a critical role for the side chain of Arg-338. This residue may function in stabilizing the conformation about the Gly-Gly turn, resulting in a specific orientation of the adjacent domains relative to each other. Rotation about the ϕ or ψ bonds of either Gly-336 or Gly-337 would have a profound effect on this orientation. The data are consistent with this as a role for the Gly-Gly sequence between the regulatory and substrate binding domains of PGDH.

D-3-Phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95)[1] from *Escherichia coli* is allosterically regulated by L-serine, the end product of its metabolic pathway (*1*, *2*). The enzyme is a tetramer of identical subunits that contains four active sites and four effector binding sites (*3*). Each subunit is made up of three distinct globular domains referred to as the regulatory, substrate binding, and nucleotide binding domains. The subunit contacts are made between pairs of adjacent regulatory domains and pairs of adjacent nucleotide binding domains to form an oval-shaped tetramer. The substrate binding domains do not make intersubunit contacts but are found between the regulatory and nucleotide binding domains in each subunit. As such, the substrate binding domains form contact interfaces with their respective regulatory and nucleotide binding domain neighbors. It has been proposed that the substrate binding domain may function in a domain shuttle mechanism that acts to transfer the signal from serine binding at the regulatory domain to the active site, which is in a cleft formed between the nucleotide binding domain and the substrate binding domain (*3*). The covalent connection between the regulatory domain and the substrate binding domain is a single polypeptide chain and that between the substrate binding domain and the nucleotide binding domain consists of only two strands of polypeptide. These regions may serve as hinge regions for movement of the adjacent domains relative to each other, as has been seen in other proteins (*4*−*6*). Such hinge regions often contain one or more glycine residues that contribute to the flexibility of the hinge by virtue of their lack of a side chain. Inspection of the putative hinge regions in PGDH shows that each contain a Gly-Gly sequence (Figure 1). In fact, the only Gly-Gly sequences in PGDH are found within these polypeptide connections. This investigation describes the results of site-directed mutagenesis in the region of one of these Gly-Gly sequences, that found at the juncture between the regulatory domain and the substrate binding domain.

MATERIALS AND METHODS

PGDH was expressed in *E. coli* and isolated as previously described (*7*, *8*). Activity was determined at 25 °C in 20 mM Tris buffer at pH 7.5 with α-ketoglutarate as the substrate by monitoring the decrease in absorbance of NADH at 340 nm (*9*, *10*). Protein concentration was determined by the Bradford method as previously described (*11*, *12*). All mutations are constructed in $PGDH_{4C/A}$, which is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine (*11*). Mutagenesis was performed as previously described (*13*) and confirmed by DNA sequencing. All PCR reagents were obtained from Perkin-Elmer. PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc.), restriction fragments were

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[1] Abbreviations: PGDH, phosphoglycerate dehydrogenase; NADH, nicotinamide adenine dinucleotide hydride; PCR, polymerase chain reaction.

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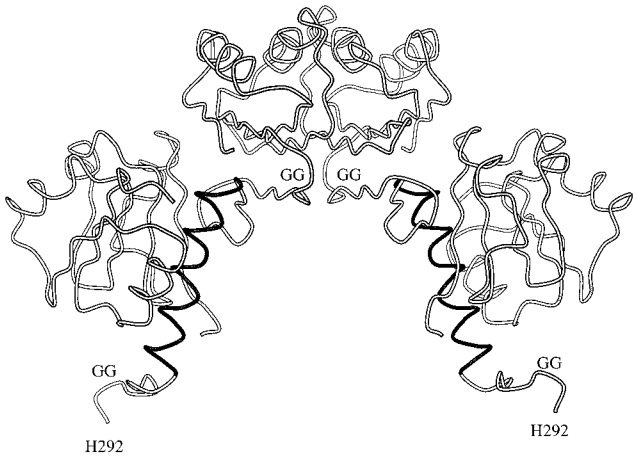

FIGURE 1: α-Carbon chain tracing of PGDH depicting the location of the two Gly-Gly sequences. Only the regulatory domains and the substrate binding domains of two subunits that interface at the regulatory domains are shown. If the NADH binding domain were present, it would be at the bottom of the figure starting at His-292. The Gly-Gly sequence that is the subject of this study is the one at the top of the figure. It is depicted in Figure 2 in more detail after a clockwise rotation of 90° in the plane of the paper. The location of the active-site histidine residue (His 292) is also shown. The α-helix that extends from the vicinity of one Gly-Gly sequence to the other is shaded black.

isolated from agarose gels with a QIAquick gel extraction kit (Qiagen Inc.), and plasmids were isolated with a QIAprep spin miniprep kit (Qiagen Inc.). All mutations were confirmed by sequencing on an Applied Biosytems Model 373 or 377 automated DNA sequencer using Big Dye terminator chemistry. PCR products were placed into plasmids by way of flanking restriction sites and the entire length of the restricted PCR insert was verified by sequence analysis. The IC_{50} value for L-serine is the concentration of serine that produces a 50% inhibition of the enzyme activity. Kinetic parameters were determined from direct linear plots (*14*). Figures 1−3 were produced with MOLSCRIPT (*15*).

RESULTS

Description of the Regulatory Domain−Substrate Binding Domain Interface. Figures 1−3 show the location of the regulatory domain Gly-Gly sequence within the context of the adjacent structural domains of PGDH as well as residues that appear to participate in potential domain−domain interactions. Gly-336 and Gly-337 are found at the apex of the turn of the single polypeptide strand that joins the regulatory domain to the adjacent substrate binding domain. The two legs of the turn are sufficiently far apart that there is virtually no interaction between main-chain atoms (Figure 2). Thus, the polypeptide segment containing Gly-336 and Gly-337 appears to be a very good candidate for the location of a relatively facile rotation about their polypeptide bonds.

The only apparent interaction between the regulatory domain and the substrate binding domain are hydrogen-bonding interactions between the guanidino groups of Arg 339, Arg, 405, and Arg 407 on the regulatory domain and main-chain carbonyl groups on the substrate binding domain (Figure 3). Specifically, the guanidino group of Arg-339 forms hydrogen bonds with the main-chain carbonyls of Leu-332 and Asn-318, the guanidino group of Arg-407 forms a hydrogen bond with the main-chain carbonyl of Ser-316, and

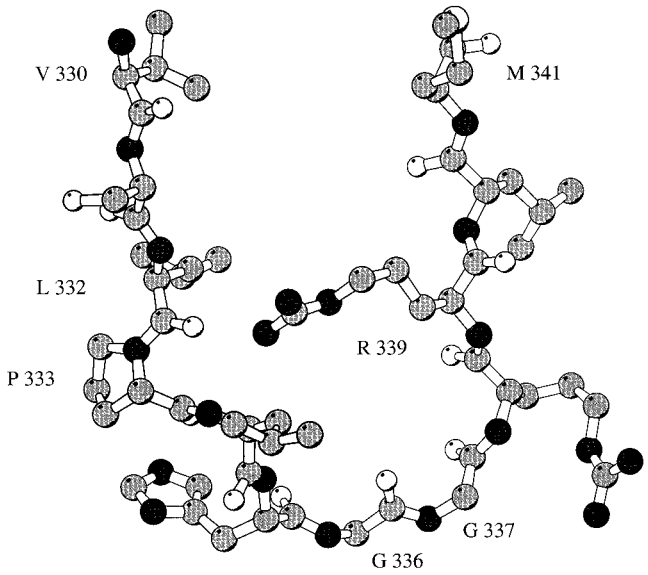

FIGURE 2: Ball and stick depiction of the turn (residues 330−341) between a regulatory domain and a substrate binding domain in PGDH. This orientation is a clockwise rotation of 90° in the plane of the paper relative to Figure 1.

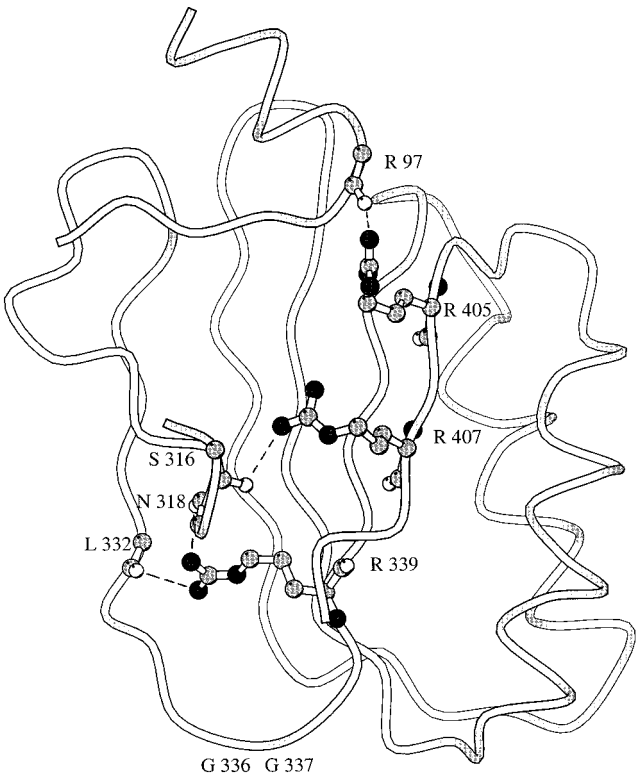

FIGURE 3: α-Carbon chain tracing of a regulatory domain and a portion of the adjacent substrate binding domain which depicts, in ball-and-stick representation, the location of proposed critical residues in the interaction between the two domains. The complete arginine side chains are shown but only the α-carbon and main-chain carbonyls of the other residues to which they are hydrogen-bonded are shown for clarity. Hydrogen bonds are depicted with a dashed line.

the guanidino group of Arg-405 forms a hydrogen bond with the main-chain carbonyl of Arg-97. Of particular interest are the hydrogen bonds from Arg-339 and Arg-407 in the regulatory domain to Asn-318 and Ser-316 in the adjacent substrate binding domain. The latter two residues are at the beginning of an α-helix that leads directly to the active-site

Table 1: Mutations at the Regulatory/Substrate Domain Interconnecting Sequence

mutant	residue number 335	336	337	338	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	IC_{50} Ser (μM)
4 C/A [a]	H	G	G	R	0.6	31.3	5.2 X 10^4	10
G337A	H	G	A	R	0.3	14.4	4.8 X 10^4	12
G336A	H	A	G	R	0.25	12.6	5.0 X 10^4	40
G336A/G337A	H	A	A	R	0.15	10.9	7.3 X 10^4	30
G337V	H	G	V	R	0.2	7.1	3.6 X 10^4	15
G336V	H	V	G	R	0.4	20.9	5.2 X 10^4	142
G336V/G337V	H	V	V	R	0.1	9.3	9.3 X 10^4	800
G337L	H	G	L	R	0.7	7.1	1.0 X 10^4	8
G336L	H	L	G	R	0.15	10.1	6.7 X 10^4	110
G336L/G337L	H	L	L	R	0.15	10.2	6.8 X 10^4	500
H335G/G336V/G337V	G	V	V	R	0.25	9.0	3.6 X 10^4	8
H335G/G337V	G	G	V	R	1.0	11.1	1.1 X 10^4	3.5
G336V/G337V/R338G	H	V	V	G	0.25	8.5	3.4 X 10^4	3000
G336V/R338G	H	V	G	G	0.15	9.9	6.6 X 10^4	3500
R338G	H	G	G	G	0.15	9.3	6.2 X 10^4	420

[a] PGDH $_{4C/A}$ is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine (*11*). It serves as the control in these experiments.

cleft (His-292, Gly-294, Gly-295). This helix is a potential route for the transmission of conformational information from the regulatory domain to the active site.

Mutagenesis of the Gly-Gly Turn. To explore their role in the communication between the effector binding sites in the regulatory domains and the active sites in the cleft between the substrate and nucleotide binding domains, site-directed mutagenesis was performed on residues 335−338. These mutants were then measured for their ability to catalyze the reaction and be inhibited by the effector L-serine. The results are listed in Table 1.

First, the role of the two glycine residues was investigated by producing mutants with side chains of increasing bulk at these positions. This was done by systematically replacing glycine with alanine, valine, and leucine residues. These mutations had little effect on the k_{cat}/K_m value, the largest difference being only 5-fold. On the other hand, some mutants had significant effect on the ability of serine to inhibit enzymatic activity.

Adding a methyl group to the α-carbon by mutating each glycine to an alanine had only a marginal effect. Among the two glycine residues, only the alteration at position 336 produced a noticeable effect by slightly reducing the ability of serine to inhibit the enzyme. Increasing the size of the side chain to that of valine or leucine had a much more profound effect. Again, in both cases, an increased effect on serine inhibition was limited to position 336 for single mutations. The single mutations at position 337 were without a noticeable effect on the IC_{50} of L-serine. Also, with both valine and leucine, the effect of mutating both glycines in the same molecule was more than merely additive. An approximate 5-fold and 50−60-fold increase in IC_{50} is seen for both double mutants compared to the single mutant at position 336 and the single mutant at position 337, respectively.

Next, the ability of the flanking positions to relieve the effect of the increasing steric bulk at the middle positions was investigated by converting each flanking residue to glycine. Again, the effect of the mutations on the k_{cat}/K_m value determined for the mutant enzymes is minimal in all cases. On the other hand, the effect of some mutations on the ability of serine to inhibit the enzyme are quite striking, ranging up to 350-fold when mutations to both central and flanking residues are combined. Interestingly, the flanking mutations had completely opposite effects depending on which side of the Gly-Gly sequence they were on. Converting the His-Val-Val-Arg sequence to Gly-Val-Val-Arg completely restored the ability of serine to inhibit the mutant enzyme to that of the unmutated enzyme. This effect was enhanced even further when position 336 was restored to glycine (Gly-Gly-Val-Arg).

On the other hand, converting Arg-338 to glycine greatly increased the IC_{50} for L-serine. When both native glycines were present (His-Gly-Gly-Gly), the increase of the R338G mutant over the unmutated enzyme was approximately 40-fold. When both native glycines were replaced by valine residues (His-Val-Val-Gly), the increase in IC_{50} was 300-fold. Furthermore, this effect was not lessened by restoring a Gly-Gly sequence that was shifted one residue to the right (His-Val-Gly-Gly).

DISCUSSION

The mutagenesis data clearly establish the single polypeptide strand connecting the regulatory and substrate binding domains as a critical link in the transmission of information from the serine binding sites to the active sites. Furthermore, the negative effect of increasing bulk on the side chains at positions 336 and 337 is consistent with a role for rotation around these polypeptide bonds as a means of transferring this signal. Gly-336 appears to be the critical residue in this mechanism since single mutations at Gly-337 have little effect. However, a side chain at position 337 can exacerbate the effect of a side chain at position 336, probably by providing additional steric hindrance to rotation at position 336.

The notion of a freely rotatable glycine residue as the underlying principle in this process is strengthened by the observation that placing a glycine at position 335 completely overcomes the effect of steric bulk at position 336. Moreover, glycines at both positions 335 and 336 produce a mutant that is even more sensitive to serine than the native enzyme.

A surprising result of these studies is the apparent requirement for the guanidino group of Arg-338 in serine inhibition. Arg-338 is exposed to solvent and appears to make

only a single hydrogen-bonding contact, that is, with the main-chain carbonyl of Ala-385. Rotation about the ϕ or ψ bonds of Gly-336, or Gly-337 for that matter, would not affect the positions of this hydrogen bond. It may be that the role of this hydrogen bond is to stabilize the position of Arg-339 (see Figure 2).

Rotation about the ϕ or ψ bonds of either Gly-336 or Gly-337, or both, would have a profound effect on the positions of Arg-339, Arg-407, and Arg-405 in relation to the substrate binding domain and to the α-helix leading to the active site. Such a rotation would make or break the hydrogen-bonding interactions of the regulatory domain with the substrate binding domain. Thus, a scenario could be envisioned where the binding of serine at the interface between the two regulatory domains may cause a conformational change about the Gly-Gly hinge that moves Arg-339, Arg-407, and Arg-405 relative to the substrate binding domain and into hydrogen-bonding contact with it. In the absence of serine binding, this interaction may not take place and the active site would be unaffected. The role of these residues within this context is presently being investigated.

If this is the link between the effector site and the active site, it could occur through global positioning of the substrate binding domain to the regulatory domain or through transmission of a signal along the α-helix that links this area to the active site. These data do not allow that distinction to be made but they clearly support a role for the residues in the turn between the two domains in the transmission of information from the serine binding sites to the active sites.

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