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A model for the regulation of D-3-phosphoglycerate dehydrogenase, a V_{max}-type allosteric enzyme

GREGORY A. GRANT,[1] DAVID J. SCHULLER,[2] AND LEONARD J. BANASZAK[3]

[1] Departments of Medicine & Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri
[2] Department of Molecular Biology & Biochemistry, University of California-Irvine, Irvine, California
[3] Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota

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Abstract

Escherichia coli D-3-phosphoglycerate dehydrogenase (ePGDH) is a tetramer of identical subunits that is allosterically inhibited by L-serine, the end product of its metabolic pathway. Because serine binding affects the velocity of the reaction and not the binding of substrate or cofactor, the enzyme is classified as of the V_{max} type. Inhibition by a variety of amino acids and analogues of L-serine indicate that all three functional groups of serine are required for optimal interaction. Removing or altering any one functional group results in an increase in inhibitory concentration from micromolar to millimolar, and removal or alteration of any two functional groups removes all inhibitory ability. Kinetic studies indicate at least two serine-binding sites, but the crystal structure solved in the presence of bound serine and direct serine-binding studies show that there are a total of four serine-binding sites on the enzyme. However, approximately 85% inhibition is attained when only two sites are occupied. The three-dimensional structure of ePGDH shows that the serine-binding sites reside at the interface between regulatory domains of adjacent subunits. Two serine molecules bind at each of the two regulatory domain interfaces in the enzyme. When all four of the serines are bound, 100% inhibition of activity is seen. However, because the domain contacts are symmetrical, the binding of only one serine at each interface is sufficient to produce approximately 85% inhibition. The tethering of the regulatory domains to each other through multiple hydrogen bonds from serine to each subunit apparently prevents the body of these domains from undergoing the reorientation that must accompany a catalytic cycle. It is suggested that part of the conformational change may involve a hinge formed in the vicinity of the union of two antiparallel β-sheets in the regulatory domains. The tethering function of serine, in turn, appears to prevent the substrate-binding domain from closing the cleft between it and the nucleotide-binding domain, which may be necessary to form a productive hydrophobic environment for hydride transfer. Thus, the structure provides a plausible model that is consistent with the binding and inhibition data and that suggests that catalysis and inhibition in this rare V_{max}-type allosteric enzyme is based on the movement of rigid domains about flexible hinges.

Keywords: enzyme control; multidomain enzyme; phosphoglycerate dehydrogenase; velocity-modulated allosterism

D-3-Phosphoglycerate dehydrogenase (PGDH) is a member of a newly recognized homologous family of oxidoreductases (Grant, 1989). At least seven members of this family are now known (Table 1; Goldberg et al., 1994), but PGDH is the only member that displays allosteric regulation. Most members of the family are 2-hydroxyacid dehydrogenases with specificity for the D-isomer of their substrate. Enzymes that catalyze reactions with L-2-hydroxyacids, such as L-lactate dehydrogenase or L-malate dehydrogenase, do not have any significant sequence similarity to these D-specific enzymes.

PGDH from *Escherichia coli* (ePGDH) is an allosterically regulated enzyme controlled by changes in V_{max}. This is in contrast to more commonly encountered regulation through changes in K_m. As affected by the presence of serine, ePGDH resembles the glycerol kinase:III ^{glc} protein complex in this V_{max} type of regulation (Hurley et al., 1993). ePGDH exists as a tetramer of identical subunits, each with a molecular weight of approximately 44,000 (Rosenbloom et al., 1968; Winicow & Pizer, 1974; Tobey & Grant, 1986). Both the catalytic and regulatory sites are part of the same polypeptide chain (Tobey & Grant, 1986; Schuller et al., 1995). The enzyme displays normal Michaelis-Menten ki-

Reprint requests to: Gregory A. Grant, Department of Molecular Biology and Pharmacology, Box 8103, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, Missouri 63110; e-mail: ggrant@pharmdec.wustl.edu.

Table 1. *PGDH-related dehydrogenases*

Species	Name	Subunits
E. coli	D-3-Phosphoglycerate dehydrogenase	4
Pseudomonas sp. 101	Formate dehydrogenase	2
Enterococcus faecium	Vancomycin resistance protein VanH	(2)[a]
Lactobacillus casei	D-2-Hydroxyisocaproate dehydrogenase	(2)
L. plantarum	D-Lactate dehydrogenase	2
L. helveticus	D-Lactate dehydrogenase	2
L. bulgaricus	D-Lactate dehydrogenase	2
E. coli	D-Erythronate-4-phosphate dehydrogenase	(2)
Cucumis sativus (cucumber)	D-Glycerate dehydrogenase	2
Hyphomicrobium methylovorum	D-Glycerate dehydrogenase	2

[a] Parentheses indicate that the subunit composition has not yet been confirmed directly.

netics with respect to substrate, product, and coenzyme. L-Serine, which does not interact directly with the catalytic site, is the physiological effector and displays sigmoidal inhibition kinetics.

The kinetics of the ePGDH reaction have been well characterized (Monod et al., 1965; Winicow & Pizer, 1974; Dubrow & Pizer, 1977a, 1977b). These studies indicate that several conformational changes take place during the reaction and that the rate-limiting step of the catalytic reaction is not the hydride transfer step, but rather one of the conformational changes. Both the forward and reverse reactions are inhibited by L-serine, with 50% inhibition being seen at 5–8 μM L-serine for the reverse reaction. The next best inhibitor is glycine, but it gives 50% inhibition only at 1–2 mM. Saturating amounts of serine completely inhibit both the forward and reverse reactions with only a slight effect on substrate binding (Dubrow & Pizer, 1977b). Thus, the inhibition is not competitive for the active site. Plots of reaction velocity versus effector concentration are sigmoidal, with a Hill coefficient near 2.0 (Sugimoto & Pizer, 1968a), indicating that the allosteric inhibition is cooperative, with the binding of one serine affecting at least two subunits of the tetramer.

ePGDH remains tetrameric in both the presence and absence of serine, so the cooperative inhibition cannot be due to a simple change in the quaternary state of the enzyme. All of the available evidence indicates that serine inhibits the enzyme by interfering with a conformational change that normally precedes hydride transfer (Dubrow & Pizer, 1977a, 1977b). This is true for both the forward and reverse reactions. Although the substrates are still capable of being bound to the enzyme when serine is also bound, the conformational shift and ensuing catalysis are not carried out. This is further confirmation that PGDH is a V_{max}-type allosteric enzyme and is thus unlike most allosteric enzymes, which are of the K_m type. Last of all, it is important to note that, with the exception of the glycerol kinase studies cited above, all of the structural information available on allosteric enzymes has been for the K_m type, and, generally, the conformational changes involve quaternary movements.

The crystal structure of ePGDH shows that the subunits are arranged with 222 point symmetry (Schuller et al., 1995). Each subunit consists of three domains, which have been called the nucleotide-binding domain, the substrate-binding domain, and the regulatory domain. Figure 1 shows the positioning of the domains in a subunit. Subunit:subunit interfaces occur between either the nucleotide-binding domains or the regulatory domains. The active site of each subunit is positioned in a crevice between the nucleotide-binding and substrate-binding domains (Fig. 1).

The crystal structure suggests a mechanism for the allosteric regulation of PGDH that correlates with the data presented here on the binding of L-serine and inhibition of enzyme activity by L-serine analogues. The mechanism involves a conformational change that accompanies serine binding and that ultimately alters the conformation of the active site crevice (Schuller et al., 1995). This report describes a model for V_{max}-type allosteric inhibition of ePGDH based on the crystal structure and direct binding and inhibition studies with effector molecules.

Results and discussion

Functional groups of serine responsible for allosteric inhibition

Early studies (Sugimoto & Pizer, 1968a) indicated that amino acids other than serine, such as glycine and threonine, could also inhibit the enzyme, but at significantly increased concentrations. In this study, a number of serine analogues with modified functional groups have been tested for effectiveness as allosteric inhibitors. These results are presented in Table 2.

The serine analogues fall into essentially three categories. The first group consists of compounds that involve a single alteration of serine, either complete removal of a functional group (e.g., glycine) or chemical modification of a single group (e.g., *N*-acetylserine or L-serinamide). These compounds retain inhibitory ability, but the concentration giving 50% inhibition (IC_{50}) is increased to millimolar concentrations in the range of 1–50 mM. The second group consists of compounds created by removal or modification of any two groups (e.g., glycineamide or *N*-acetyl-L-serinamide; also compare L-serinamide to glycineamide and *N*-acetyl-L-serine to *N*-acetylglycine). These changes result in a greater loss of inhibitory potency, such that no inhibition is seen at 50 mM. The last group includes compounds in which only a single functional group was removed or modified but in which inhibitory potency is nevertheless lost, probably owing to the introduction of a sterically large moiety, for example phenylalanine, or to alteration of the stereochemistry around the alpha carbon, as in the case of ethanolamine. The presence of a sub-

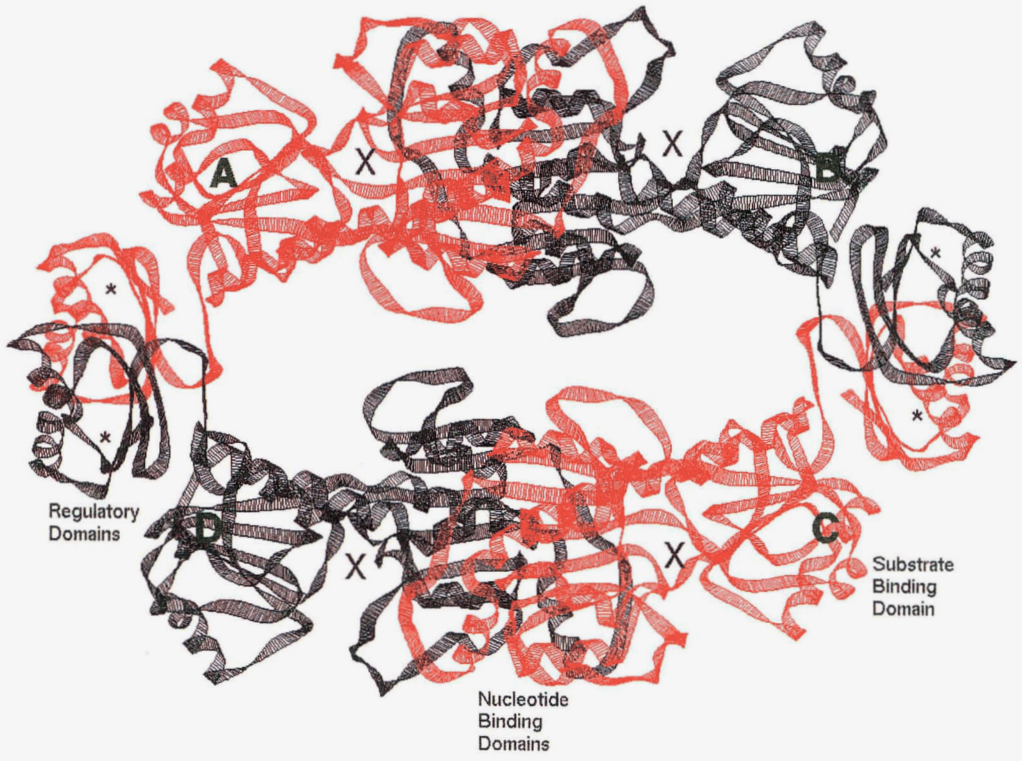

Fig. 1. The regulated unit of an ePGDH tetramer. Ribbon drawing based on the crystallographic coordinates of the PGDH tetramer. Crystalline PGDH has 222 point symmetry; this view is looking down one of the dyads, with the other symmetry axes running horizontally and vertically through the center. The subunits are labeled A–D, and the three domains making up a subunit are labeled "regulatory," "nucleotide-binding," and "substrate-binding." For clarity's sake, every domain in each of the four subunits has not been labeled, but their identification should be obvious from the visible symmetry. The single polypeptide strand connecting the regulatory and substrate-binding domains is visible, but elements of secondary structure that protrude behind the plane of the figure obscure the two segments of connecting polypeptide between the substrate-binding domain and the nucleotide-binding domain. The active site in each subunit is marked by a large X; serine (allosteric) sites are marked by asterisks. Note that each pair of contacting regulatory domains (of subunits A and D or B and C) forms a continuous β-sheet (shown in more detail in Fig. 2). This tight subunit:subunit interface, along with close packing between the contacting nucleotide-binding domains (of subunits A and B or C and D), are the principal interactions that stabilize the tetramer. It should be clear that the effect of serine at the allosteric site must be transmitted to the distant active site. Actual serine-binding sites are shown in Figure 2 using the same color coding. Drawing prepared with MIDAS software (Ferrin et al., 1988).

stituent with a bulky side chain may affect the inhibitor's ability both to get to the binding site and to fit in the same manner as serine. The loss of an asymmetric alpha carbon in ethanolamine, although this compound retains two of the three functional groups, is consistent with the observation that D-serine also is not an effective inhibitor of the enzyme (Pizer, 1963). Our observations with D-serine confirm that it does not inhibit in the low micromolar range. However, D-serine was not included in these studies because of the technical difficulty in obtaining it free from L-serine contamination. Only a few percent contamination by L-serine would be enough to produce substantial inhibition at the concentrations of D-serine required. Because only L-serine shows micromolar binding constants, these data suggest that at least two of the three functional groups of L-serine are required for inhibition, and that all three are necessary for optimal inhibition.

As predicted by the crystallographic model, the reduced potency of L-homoserine suggests that there are fairly tight constraints on the position of the aliphatic hydroxyl group. In addition, the progressively weaker inhibition seen with molecules lacking a polar group on the side chain (glycine, alanine, α-aminobutyrate, and norvaline) is consistent with a polar environment in the vicinity of the serine side chain. A comparison of the concentration dependence of activity for L-threonine and L-allothreonine indicates that the orientation of the β-hydroxyl also has an effect. Although L-cysteine differs from L-serine only by the presence of sulfur in place of oxygen, it is not unexpected that it is a poor inhibitor, because the sulfhydryl group is generally a poor hydrogen bond donor. In addition, the van der Waals volume of sulfur is approximately twice that of oxygen, so that steric effects alone could account for the difference. From the chemical results presented here, it appears that stereospecific interactions of the three polar groups determine the affinity and therefore the effectiveness in catalytic inhibition. Removal or modification of one of the polar interactions noticeably reduces the affinity of the inhibitor. However, binding appears to be possible if any two of the potential interactions are present in the proper orientation and the concentration of effector is sufficiently elevated. This model also suggests that at least one of the L-serine functional groups may form hydrogen bonds with both regulatory domains at the interface.

The hydrogen bonding pattern at the bound serine site as determined from the crystal structure is shown in Figure 2. All three polar groups of the bound L-serine are involved in hydrogen

Table 2. *Inhibition of E. coli PGDH*

Reagent	IC_{50} (mM)[a]	Reagent	IC_{50} (mM)[a]
L-Serine	0.008	L-Threonine	27.0
O-acetyl-L-serine	1.4	*N*-α-benzyl-L-serine	50.0
L-Allothreonine	1.5	*N*-acetyl-L-serinamide	N[b]
Glycine	1.8	*N*-acetylglycine	N
L-Alanine	3.0	Glycineamide	N
L-Serinamide	3.8	L-Norvaline	N
L-Cysteine	4.1	L-Norleucine	N
L-Homoserine	5.1	*O*-benzyl-L-serine	N
N-acetyl-L-serine	5.2	L-Phenylalanine	N
N-Cbz-L-serine	15.0	L-Glutamine	N
L-α-Aminobutyrate	18.5	Ethanolamine	N

[a] Concentration producing 50% inhibition.
[b] No inhibition at 50 mM.

bonds with the enzyme and, taken together, they form a hydrogen bonding network that spans the domain:domain interface. The network also involves the two water molecules shown in Figure 2, which, in addition to interacting with the serine side-chain oxygen, also are within hydrogen bonding distance of each other. Furthermore, they are also within hydrogen bonding distance of protein atoms on each of the two subunits. The protein atoms linked to these water sites include the amide nitrogen of T352 from the D-subunit and the carbonyl oxygen of V363 of the A subunit. T352 and V363 both belong to helices near each other in the regulatory domain interface (see Fig. 1).

Stoichiometry of serine binding

Early kinetic studies (Sugimoto & Pizer, 1968a) indicated that ePGDH possessed at least two effector sites. Because it is a tetramer of identical subunits, this meant that either two subunits came together to form a single site or that, although each subunit contained a single site, occupancy of only two sites was sufficient for nearly complete inhibition. In the crystal structure, there are four serine sites, and each appears to be equally occupied. However, the crystal structure offers no insight into the level of occupancy necessary for complete inhibition.

To clarify this situation, equilibrium dialysis binding studies were performed with radiolabeled L-serine. The data, in the form of Scatchard and Hill plots, are presented in Figure 3. At low serine concentrations, positive cooperativity of binding is evident, followed by an apparent negative cooperativity at higher serine concentrations. Hill coefficients for the two sites are 1.5 and 0.85, respectively. Overall, the data in Figure 3 indicate that there are a total of four binding sites for serine on ePGDH, of which two are "tight" binding sites. The dissociation constant for the "tight" binding sites is approximately 5 μM. This corresponds closely to the value of 5–8 μM for the concentration of serine that produces 50% inhibition.

Inspection of the data for percent inhibition of the enzyme as a function of sites occupied, shown in Table 3, indicates that the enzyme is approximately 85% inhibited when only two sites

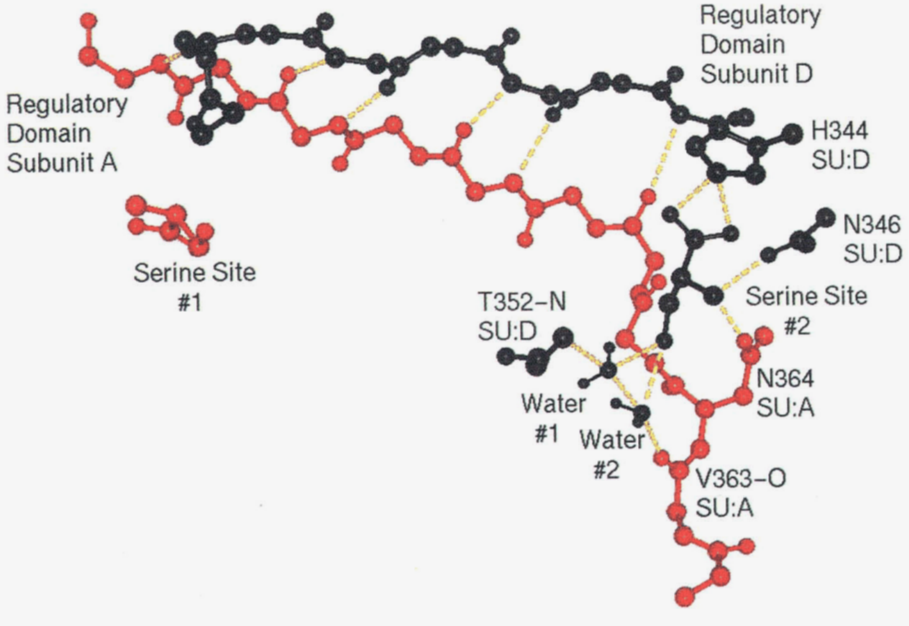

Fig. 2. Hydrogen bonding between L-serine and the regulatory domains of adjacent subunits. The drawing shows the interface between the regulatory domains in the ePGDH tetramer. Color coding is as in Figure 1: red, A subunit; black, D subunit. Yellow dotted lines represent hydrogen bonds, defined by distances between polar atoms ranging from 2.6 Å to 3.3 Å. The allosteric effector serine is present at two sites in the interface, which are related by a twofold rotation axis. Only one serine site is shown in detail. Note that the serines are actually located below the plane of the β strands in this figure (see Fig. 1). The two water sites near the -OH of bound serine, labeled "Water #1" and "Water #2," interact, respectively, with the peptide nitrogen of Thr 352 on subunit A and the carbonyl oxygen of Val 363 or subunit D. In this crystallographic model, the α-amino group of the bound serine forms hydrogen bonds with side-chain atoms of Asn 364 of subunit A and Asn 346 of subunit D. β-Strands from each of the two subunits form six interstrand hydrogen bonds. Drawing prepared with MIDAS software (Ferrin et al., 1988).

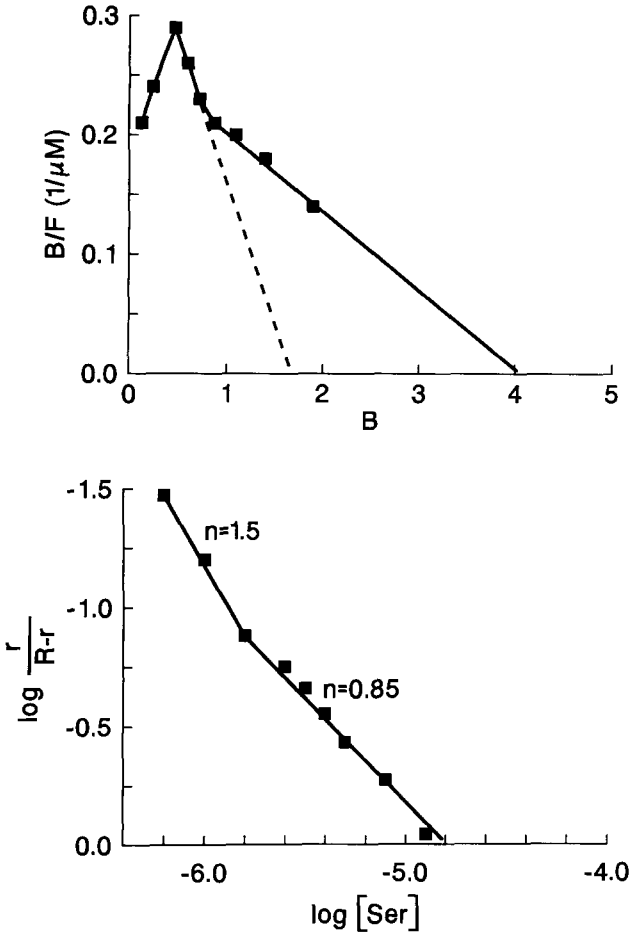

Fig. 3. Binding of L-serine to PGDH. Binding of L-serine is shown in both a Scatchard plot (top) and a Hill plot (bottom).

are occupied. This result is consistent with the earlier kinetic observations. Occupancy of the remaining two sites occurs only at concentrations of serine significantly higher than those required for the first two sites, and results in complete inhibition of activity. Inclusion of glycine at a concentration that completely inhibits the enzyme results in effective competition with serine for the binding sites, which confirms that serine and glycine bind at the same sites.

Table 3. *Site occupancy versus inhibition*

Serine (μM)	Sites occupied	% Inhibition
1	0.25	0
3	0.70	13
5	1.0	33
6.3	1.2	54
8	1.5	73
12	1.8	81
15	2.0	84
50	3.0	96
5.5 + 50 mM glycine	0.2	>95
9.5 + 50 mM glycine	0.4	>95

If one assumes, as a first approximation, that the difference in concentration dependence for inhibition corresponds approximately to the difference in K_d, the difference in the inhibitory capacities of serine and glycine (8 μM and 2 mM, respectively) corresponds to a change in standard free energy of binding of approximately 3.5 kcal/mol. This correlates well with the average energy of a hydrogen bond (2.5–4 kcal/mol) and with the loss of one or two hydrogen bonds when serine is replaced with glycine.

Relationship of binding and inhibition data to the structure of ePGDH

The crystal structure of ePGDH, along with the location of active sites and the intersubunit location of the effector sites, is shown in Figure 1. Each subunit of the tetramer is clearly divided into a nucleotide-binding domain, a substrate-binding domain, and a regulatory domain. The substrate-binding domain consists of residues 7–107 and 295–336, and the nucleotide-binding domain consists of residues 108–294. Residues 2–6 were not visible in the electron density map (Schuller et al., 1995). Thus, the polypeptide chain first forms part of the substrate domain, then the entire nucleotide domain, then the remainder of the substrate domain. The regulatory domains comprised of the remaining C-terminal residues, 337–410.

The nucleotide and regulatory sites of PGDH are clearly identified because the crystal structure was solved with NAD and serine bound to their respective domains (Schuller et al., 1995). The locations of these sites are given in Figure 1, where they are called the active site and the serine-binding site. The substrate-binding location is inferred from the presence of an anion-binding site adjacent to the NAD cofactor and the conserved catalytic residues (Schuller et al., 1995). This anion site is assumed to be the site of the phosphate moiety of the substrate and is centered about Arg 60. A conserved residue in the nucleotide-binding site, Arg 240, may also bind to the carboxylic acid moiety of the substrate (Schuller et al., 1995).

As a result of the domain structure, the only covalent connections between the nucleotide and substrate domains are two short segments of polypeptide chain adjacent to each other and close to the NAD-binding site and the catalytically active residues. (This feature is not clear in Fig. 1 because elements of secondary structure protruding behind the plane of the figure obscure the two segments of connecting polypeptide.) The covalent connection between the substrate-binding domain and the regulatory domain is only a single segment of polypeptide chain, clearly visible in Figure 1. Although the steric relationships of the domains are fixed in the tetramer, we perceive the connecting segments of polypeptide chains to be flexible. Such flexibility would make possible variations in the relative domain:domain orientations.

To add substance to the hypothesis that domain:domain variations are the source of allosteric effects, it is important to consider the domain:domain and the subunit:subunit interfaces. The nucleotide-binding domains of subunits A and B (and, by symmetry, C and D) form a close contact to make up one of the subunit:subunit interfaces of the tetramer (Schuller et al., 1995). This extensive interface covers 2600 Å^2 and is preserved in the homologous enzymes glycerate dehydrogenase (Goldberg et al.,

1994) and formate dehydrogenase (Lamzin et al., 1994). The large area of this interface and its conservation in nonallosteric enzymes lead us to believe that this subunit interface is stable and rigid.

The regulatory domains of subunits A and D (and, by symmetry, B and C) form the only other significant subunit:subunit interface in the tetramer (Schuller et al., 1995). The regulatory domain of ePGDH is unique and distinguishes it from glycerate and formate dehydrogenase. The ePGDH regulatory domain consists of four antiparallel β-strands connected by two α-helices and one turn. Although of unknown significance, the motif of the ePGDH regulatory domain is found in several other proteins, including nucleoside diphosphate kinase and the regulatory domain of aspartate carbamoyltransferase (Schuller et al., 1995). In all three proteins, the subunit interfaces form an extended eight-stranded β-sheet.

In spite of the similarity in the way that they form a dimer interface, only in ePGDH are the allosteric sites located between two regulatory domains. Hence, in this allosteric system, the effector molecule forms a hydrogen bonding network that effectively adds to the noncovalent linkage between the two subunits, as shown in Figure 2. The crystal structure indicates that the serine carboxylate interacts with the imidazole ring of His 344, whereas the serine amino group forms a hydrogen bonding network with the side chains of Asn 346 and Asn 364′ in the adjacent subunit (Schuller et al., 1995).

As can be seen in Figure 2, the crystal structure does not show a direct link between the protein and the -OH of the bound serine. However, two water molecules are within hydrogen bonding distance both of this -OH and of protein atoms in the two subunits. As can be seen in Figure 2, water molecule #1 interacts with the peptide nitrogen of Thr 352 (subunit D), and the water molecule #2 is hydrogen bonded to the carbonyl oxygen of Val 363 (subunit A). In addition, the backbone nitrogen of Ile 365′ and the carbonyl oxygen of Arg 347 are nearby.

Thus, two bound serine molecules appear to link the adjacent regulatory domains through a network of hydrogen bonds to each subunit. As can be seen in Figure 1, the serine sites are located approximately midway between the β-sheet and the four-membered α-helical layer formed by the union of the two adjacent regulatory subunits. The helical and sheet layers are visible in Figure 1 to the left and right, respectively, of the serine-binding sites.

The hydrogen bonding network when serine is present at the regulatory interface is consistent with the binding and inhibition data presented here. Removing or altering any one functional group of serine eliminates at least one or two hydrogen bonds but still leaves some subunit-to-subunit crosslinking. This state is consistent with a decrease in IC_{50} by approximately three orders of magnitude. Removal of two functional groups generally eliminates the molecule's ability to mediate sufficient subunit-to-subunit interaction and thus its ability to cause inhibition. We propose that the crosslinking nature of the hydrogen bonds between serine and the two regulatory domains is probably at the root of the regulatory mechanism.

The effector sites are distant from both each other and the active site. At a regulatory subunit interface, the effector sites are 17 Å apart and more than 100 Å from the other two bound serine molecules in the tetramer. The nicotinamide ring of the NAD at the active site is approximately 33 Å from the closest serine, thus eliminating any direct steric effect on the active site. All four serine sites are fully occupied in the crystal structure, and the interface between the two regulatory sites is completely closed so that the serine molecules are buried in the interface and not easily accessible to solvent. Either a conformational change involving part of a regulatory subunit must occur or the interface between the two regulatory subunits must open and close to allow serine access to its binding sites. In the latter case, a movement of the interface to accommodate this opening has been proposed (Schuller et al., 1995) and is elaborated below. Alternatively, because of the position of the serine-binding sites between the layer of β-sheet and α-helices in the regulatory domain interface, another mechanism might involve small changes in the distance between the continuous sheet and the helical layer.

The allosteric changes associated with serine binding must be transmitted to the active sites of the enzyme. A possible mechanism for this chemical effect is as follows. The relative locations of NAD and the inferred substrate site indicate that the active site is in a cleft between the nucleotide-binding domain and the substrate-binding domain (Fig. 1). We believe that the active site cleft observed in the crystal structure is in an "open," or inactive, state. This proposal is supported by the observation that the contact between the substrate and nucleotide domains is less than 100 $Å^2$.

To get to the "closed" or active state of the enzyme, a change must occur at the interface between the nucleotide- and substrate-binding domains. Evidence for the flexibility between these two domains is found in the crystal structure. Two crystallographically independent subunits have a difference corresponding to a 12° rotation between the nucleotide- and substrate-binding domains. Two adjacent glycines (Gly 294 and Gly 295) are found at this hinge segment and may be important to flexibility in this region.

In the "open" configuration, the cleft between the two subunits is wide, and the active site is accessible to solvent and substrate. In the "closed" configuration, not seen in the crystal structure with the inhibitor serine present, the cleft would be narrowed to provide the necessary proximity for catalysis. The closure of the active site would require domain motion that would be inhibited by the presence of serine in the regulatory domains. Thus, the binding of serine to the regulatory domain during allosteric inhibition must somehow be communicated to the active sites of the involved subunits and, clearly, the interface between the regulatory domain and the adjacent substrate-binding domain in the same subunit is crucial to the understanding of the allosteric regulation. The direction and extent of the motion required would be complex.

To further define the conformational changes involved in the regulation, structural data on the uninhibited enzyme will be needed. To date, it has not been possible to obtain good crystals of the enzyme in the absence of serine. However, in conjunction with the binding results presented above, the available crystal coordinates suggest a mechanism by which this could occur.

A model for V_{max}-type allosteric regulation in ePGDH

The kinetic studies indicate that serine inhibits the ternary complex isomerizations that normally occur during catalysis (Dubrow & Pizer, 1977a, 1977b). The binding studies presented here

show that as much as 85% inhibition occurs when only two serine molecules are bound to the tetramer, presumably one at each of the two regulatory subunit interfaces, A-D and B-C in Figure 1. The effect appears to come about through the tethering of the regulatory domains to each other at the interface by a single effector molecule. Because of the symmetry of the tetramer, two serine molecules can eventually bind at the same regulatory domain interface. The binding of a second serine at each interface strengthens the interaction such that 100% inhibition is achieved.

Because ePGDH remains a tetramer in the absence of serine, any model proposed for the allosteric mechanism may not involve complete subunit:subunit separation at the regulatory domain interface. The available data suggest that, in binding at the interface, each L-serine molecule forms four to six hydrogen bonds, two or three to each adjacent domain. The intersubunit hydrogen bonds are all shown in Figure 2. Because of the bifurcated interactions at each of the polar atoms of the effector, it is difficult to assign a precise number. For example, the polar interactions between the serine carboxylate and His 344 involve a bifurcated hydrogen bonding system, the energetics of which are unclear. Nonetheless, removing any one of the points of contact results in a decrease in binding affinity of approximately three orders of magnitude. Removing two of the three points of contact results in a loss of inhibitory ability, perhaps because the regulatory domains of the two adjacent subunits are no longer sufficiently crosslinked to maintain most of the molecules in the open-cleft active site form.

The crystal structure suggests that the cooperativity seen in serine-induced inhibition is likely to occur between the two subunits linked at their regulatory domains (A and D or B and C) (Schuller et al., 1995). The distance of the allosteric site from the active site requires that the effect of serine be transmitted through a relatively long distance. We have suggested that the two regulatory domains may move relative to one another, perhaps in a hinging motion, flexing as the binding of serine to the effector site excludes solvent water and increases the interactions between two regulatory domains.

The binding of serine, in turn, stabilizes the closed state of the allosteric site and the open state of the active site (Fig. 4). The closed state of the regulatory domains must then stabilize the substrate-binding domain, preventing it from moving toward its nucleotide-binding domain to close the active site cleft. This requires a separate mechanism for the communication of the serine binding state to the active site.

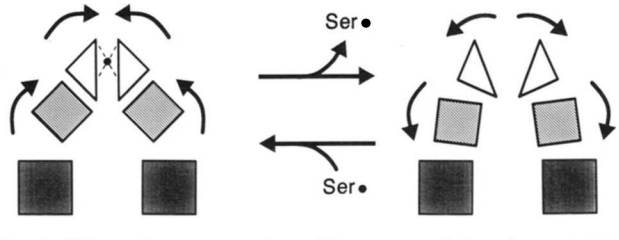

Fig. 4. Schematic representation of the proposed domain movement about flexible hinges in PGDH. The nucleotide-binding domain is depicted as a large square, the substrate-binding domain as a smaller square, and the regulatory domain as a triangle. L-Serine is depicted as a filled circle. Only two subunits are shown.

A multihinge model, or some variation of it, explains all three phenomena. The cooperativity of inhibition results from a single serine stabilizing the closed regulatory hinge state. The serine-binding data indicate that cooperativity for the third and fourth sites of a tetramer is lower than for the first two. This agrees with the proposal that a single serine is enough to close the interface. A second serine, binding at the same interface, would have reduced access to its site because the interface was closed part of the time. Finally, the domain motions of the hinge model provide a method of communicating the binding of serine at the allosteric site to the rest of the molecule.

For the hinge mechanism to work properly, neither the open nor the closed state should be so stable as to prevent ePGDH from switching from one state to the other. At the center of the regulatory domain subunit interface, where a hydrophobic core might be expected, Asn 355 and Gln 368 are found instead. These polar amino acids may serve to weaken the intersubunit contacts by accommodating solvent into the interface in a way that hydrophobic contacts would not. This may help limit the stability of the closed hinge state when serine is not bound.

Once the binding of serine closes the regulatory domain interface, that information must somehow be communicated to the active site. This points to an important role for the interface between the regulatory and substrate-binding domains. We believe this interface to be flexible. Various points of circumstantial evidence support this viewpoint. Assuming that closing of the active site cleft is necessary for catalysis, a rigid interface between the substrate-binding and regulatory domains would require cooperativity between at least two subunits for substrate binding. No such cooperativity is seen in the kinetic measurements. Furthermore, no secondary structure crosses this interface. The two domains are linked covalently by only one polypeptide strand at the far inside end of the interface, approximately at the midpoint of a loop consisting of residues 320–336, which form most of the contacts between the regulatory and substrate-binding domains.

The possible involvement of residues 320–336 in forming a contact at the regulatory domain:substrate-binding domain interface may provide an explanation of how the closure of the allosteric site could influence the interface between the regulatory and substrate-binding domains. In the inhibited state, the substrate-binding domain would be locked to the regulatory domain, preventing the substrate-binding domain from closing on the active site. In the active state, the substrate-binding domain would be freed from the regulatory domain and would be free to move about the single polypeptide connection, allowing it to function in closing and opening the active site for catalysis. This opening and closing of the active site cleft would account for the conformational shifts seen in the kinetic studies (Dubrow & Pizer, 1977a, 1977b). The extent of change occurring at the interface joining the regulatory and substrate-binding domains is not known.

Thus, the catalytic activity and allosteric regulation of ePGDH by L-serine appears to be implemented through motion of rigid domains about flexible hinges. Catalysis requires regular closing and opening between the substrate-binding and nucleotide-binding domains. The binding of serine at the allosteric site tethers the regulatory domains in a closed state. The conformation of the closed regulatory domains then locks the substrate-binding domain to the regulatory domain, preventing the motion of the substrate-binding domain that is necessary for catalysis.

Materials and methods

The ePGDH enzyme was isolated from *E. coli* as described previously (Grant & Zapp, 1981; Schuller et al., 1989). The enzyme was assayed by following the decrease in NADH at 340 nm using hydroxypyruvic acid phosphate as a substrate (Grant & Zapp, 1981; Schuller et al., 1989). L-Serine, glycine, L-alanine, L-cysteine, L-homoserine, *N*-CBZ-L-serine, L-α-aminobutyrate, L-threonine, L-norvaline, L-norleucine, *N*-acetylglycine, glycineamide, L-phenylalanine, L-glutamine, and ethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO). *O*-Benzyl-L-serine, *N*-α-benzyl-L-serine, *N*-acetyl-L-serinamide, *O*-acetyl-L-serine, L-serinamide, and *N*-acetyl-L-serine were originally purchased from Vega Biochemicals (Tucson, AZ). [^{3}H]-L-serine was purchased from Amersham (Arlington Heights, IL). All other reagents were of the highest grade commercially available.

Inhibition studies with serine analogues were performed in standard assay buffer, 50 mM Tris, pH 7.5, 2 mM DTT, and 1 mM EDTA, with saturating levels of substrate and cofactor and at room temperature. Each analogue to be tested was first dissolved in assay buffer, and the pH was adjusted if necessary. Analogues were added to the cuvette at appropriate concentrations just prior to the addition of enzyme, which initiated the reaction. For those analogs that produced inhibition, at least six concentrations were used, three on either side of the IC_{50}.

Equilibrium dialysis was performed with a Hoffler EMD 101 Microdialyzer employing an EMD 104 membrane with a molecular weight cut-off of 6,000–8,000. Binding experiments were performed in 20 mM imidazole buffer, pH 7.5, containing 1 mM EDTA and 2 mM dithiothreitol. Sample cells were analyzed by liquid scintillation counting, and protein was quantified by amino acid analysis. The standard free energy of dissociation was estimated with the standard equation $\Delta G_d^\circ = RT \ln K_d$.

The x-ray crystallographic coordinates for ePGDH are available from the Protein Data Bank at the Brookhaven National Laboratory under the heading 1PSD.

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