Protein Science (1999), *8*:2501–2505. Cambridge University Press. Printed in the USA.

The relationship between effector binding and inhibition of activity in D-3-phosphoglycerate dehydrogenase

GREGORY A. GRANT, XIAO LAN XU, AND ZHIQIN HU

Department of Molecular Biology and Pharmacology and the Department of Medicine,
Washington University School of Medicine, 660 S. Euclid Avenue, Box 8103, St. Louis, Missouri 63110

(RECEIVED June 15, 1999; ACCEPTED August 12, 1999)

Abstract

The binding of L-serine to phosphoglycerate dehydrogenase from *Escherichia coli* displays elements of both positive and negative cooperativity. At pH 7.5, ~2 mol of serine are bound per mole of tetrameric enzyme. A substantial degree of positive cooperativity is seen for the binding of the second ligand, but the binding of the third and fourth ligand display substantial negative cooperativity. The data indicate a state of ~50% inhibition when only one serine is bound and ~80–90% inhibition when two serines are bound. This is consistent with the tethered domain hypothesis that has been presented previously. Comparison of the data derived directly from binding stoichiometry to the binding constants determined from the best fit to the Adair equation, produce a close agreement, and reinforce the general validity of the derived binding constants. The data also support the conclusion that the positive cooperativity between the binding to the first and second site involves binding sites at opposite interfaces over 110 Å apart. Thus, an order of binding can be envisioned where the binding of the first ligand initiates a conformational transition that allows the second ligand to bind with much higher affinity at the opposite interface. This is followed by the third ligand, which binds with lesser affinity to one of the two already occupied interfaces, and in so doing, completes a global conformational transition that produces maximum inhibition of activity and an even lower affinity for the fourth ligand, excluding it completely. Thus, maximal inhibition is accomplished with less than maximal occupancy of effector sites through a mechanism that displays strong elements of both positive and negative cooperativity.

Keywords: allosteric regulation; dehydrogenase; NADH-dependent oxidoreductase

D-3-Phosphoglycerate dehydrogenase (PGDH) from *Escherichia coli* is a tetrameric enzyme that is inhibited in an allosteric manner by L-serine (Sugimoto & Pizer, 1968a, 1968b). The subunits are identical, and each is composed of three domains (Schuller et al., 1995). The cleft between the substrate binding domain and the nucleotide binding domain forms the active site of the enzyme. The third domain, called the regulatory domain, binds L-serine at the interface between it and an adjacent regulatory domain from a neighboring subunit. As a result of this geometry, each tetramer contains four active sites, but only two serine binding domain interfaces, which can each bind two serine molecules with 180° symmetry for a total of four serines per tetramer. Earlier studies (Al-Rabiee et al., 1996a) have shown that covalently tethering adjacent regulatory domains with engineered disulfide bonds results in inhibition of enzymatic activity in the absence of L-serine. This supported the hypothesis (Grant et al., 1996) that serine inhibited PGDH by stabilizing the association of adjacent regulatory domains with a hydrogen bonding network, and that regulatory domain movement may approximate that of rigid bodies flexing about a hinge (Al-Rabiee et al., 1996b). Additional studies (Grant & Xu, 1998) have shown that a conformational change relative to adjacent regulatory domains can be observed as a function of serine concentration by the quenching of an engineered tryptophan residue placed at the regulatory domain interface. These studies provided preliminary evidence that the binding of only a single effector molecule at each interface may be sufficient to account for the observed inhibition of activity and the fluorescence quenching. This reinforced the notion that it is the tethering of regulatory domains, and not the occupancy of the four serine binding sites per se that influence the four active sites in the tetrameric enzyme (Grant et al., 1999).

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.

Results and discussion

The inhibition of PGDH by L-serine in Tris buffer at pH 7.5 and 25° using α-ketoglutarate as substrate displays sigmoidal characteristics (Fig. 1). When these data are fit to the Hill equation, it produces a coefficient of cooperativity of ~2.0, indicating that interaction between subunits is occurring.

To explore the relationship between serine binding and activity in more detail, binding studies were performed by equilibrium

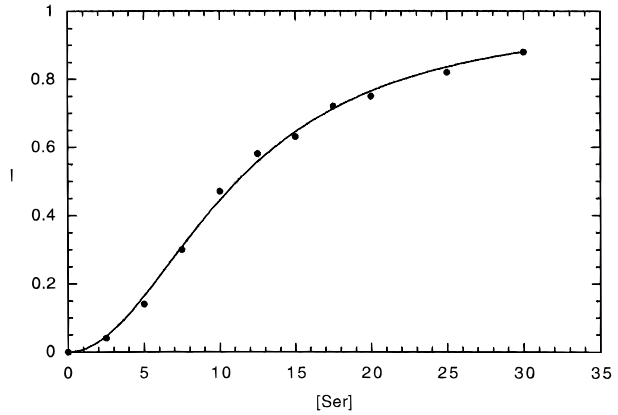

Fig. 1. L-Serine inhibition of PGDH in tris buffer pH 7.5. The fractional inhibition of enzyme activity is plotted vs. L-serine concentration (μM), ●. The line is produced by fitting the data to the Hill equation.

dialysis. The binding data along with a Scatchard analysis are shown in Figure 2. The concave nature of the Scatchard plot indicates positive cooperativity, while the binding of less than 4 mol of serine per mol of tetrameric enzyme indicates negative cooperativity. Fitting the binding data to the Adair equation (solid line in Fig. 2) yields the stepwise Adair constants shown in Table 1 and the intrinsic site dissociation constants, which can be derived from the Adair constants. The constants suggest a high degree of positive cooperativity in binding to the second site, followed by significant negative cooperativity for the last two sites, especially the fourth site.

Table 1. *Analysis of binding data*[a]

	μM		μM
K_1	$53.3 \pm 1.3 \times 10^{-4}$	K_1'	213
K_2	$2.8 \pm 2.6 \times 10^{-3}$	K_2'	4.2
K_3	$83.9 \pm 5.3 \times 10^{-5}$	K_3'	55.9
K_4	Very large	K_4'	Very large

[a]Expressed as dissociation constants. Adair constants are denoted as K_i and intrinsic site dissociation constants are denoted as K_i'.

If one assumes that the serine concentration in activity assays approximates the free serine concentration, a comparison between the extent of serine binding and the inhibition of enzymatic activity by L-serine can be made. This plot (Fig. 3) indicates a state of ~50% inhibition when only an average of one serine is bound and ~90% inhibition when two serines are bound.

Figure 4 shows a representation of the distinct ligated states for serine binding to a tetramer of four identical subunits, which is consistent with the tethered domain model. Subunits are represented by circles, and the regulatory domains are adjacent to each other in those subunits that are across from each other horizontally. Serine binding to a subunit is depicted by a filled circle, and an arrow is used to indicate that the serine also contacts the adjacent regulatory domain via hydrogen bonds. For a tetramer of four identical subunits, the 16 possible states reduces to 7 (states 0–4 plus 2a and 2b). For those states with two ligands bound (2, 2a, 2b), the two states with one ligand bound at each interface (2 and 2b) differ only by the relative orientation of ligand in the binding sites. If the domain connectivity is the crucial element and orientation of the ligand is not an issue, then in effect, this reduces the number of possible functional states to six (0–4 plus 2a).

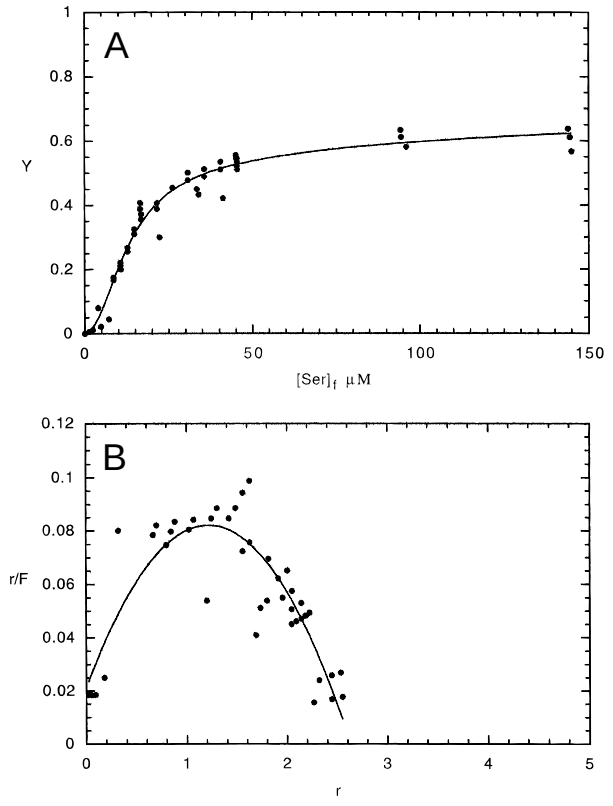

Fig. 2. L-Serine binding to PGDH. **A:** The fractional occupancy of the tetramer (Y) is plotted vs. the free L-serine concentration (μM). The solid line is the data fit to the Adair equation for four binding sites. The correlation coefficient for the fit is $R = 0.994$. **B:** Scatchard plot of the binding data, where r is the number of serines bound per tetramer and r/F is r divided by the free serine concentration.

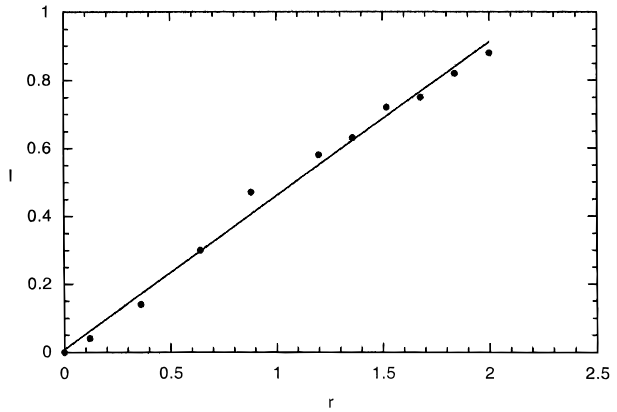

Fig. 3. Relation of bound serine to degree of inhibition of PGDH. Mol of serine bound per mol of tetramer (r) is plotted against the fractional inhibition of the enzyme.

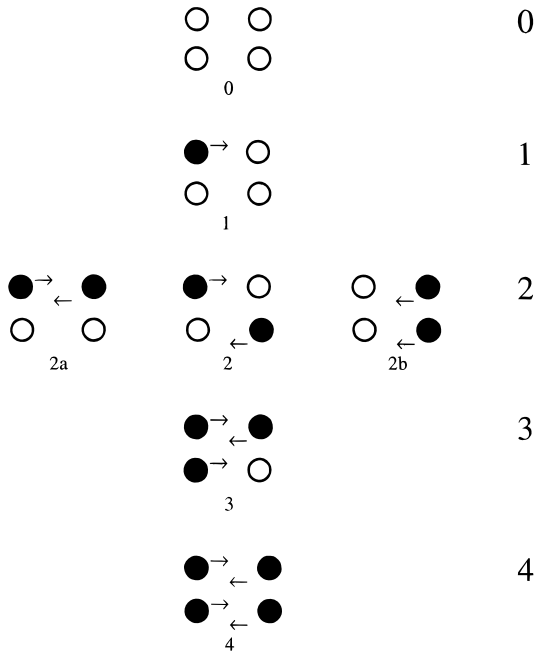

Fig. 4. Distinct ligated states for the interaction of serine with PGDH. The circles represent the four identical subunits of the PGDH tetramer. Filled circles indicate that the serine binding site for that subunit is occupied in a specified orientation. The arrows indicate that the bound serine is forming hydrogen bonding contacts with the adjacent subunit. The combinations from 0–4 bound serines are shown. The symmetry of the tetramer reduces the number of states from 16 to 7.

A strict interpretation of the tethered domain hypothesis would say that if the bound serine influences the active sites of both subunits to which it is hydrogen bonded, then when one serine is bound, approximately one-half of the active sites of the tetramer should be affected. From this it follows that approximately the same degree of inhibition will be seen when two serines are bound at the same interface as when only one serine is bound (states 1 and 2a in Fig. 3). Maximum inhibition will be observed when at least one subunit in each of the two horizontal pairs has serine bound. In this case, the degree of inhibition is not expected to increase substantially with the binding of the third and fourth ligand.

To further evaluate the data, the fractional distribution of species with bound serine was calculated from the Adair constants (Fig. 5). This plot provides a visual representation of the effects of the positive and negative cooperativity, and allows an evaluation of the relationship between incremental site occupancy and inhibition based on the determination of the binding constants. This plot shows that binding of the second ligand occurs almost simultaneously with the binding of the first so that the species with one serine bound is always very low and the species with two serines bound predominates (Fig. 4). If each of the first two serines bind at opposite interfaces, the inhibition of activity that results would be expected to be predicted by $I \approx (0.5P_1 + P_2 + P_3 + P_4)$. If the first two serines bind at the same interface, the inhibition pattern would be predicted by $I \approx (0.5P_1 + 0.5P_2 + P_3 + P_4)$. Figure 6 shows that there is good agreement between the inhibition profile and the serine occupancy calculated from the distribution profile shown in Fig. 5 using $I \approx (0.5P_1 + P_2 + P_3 + P_4)$.

Perhaps the most striking feature of the binding is the large swing between apparent positive and negative cooperativity. The

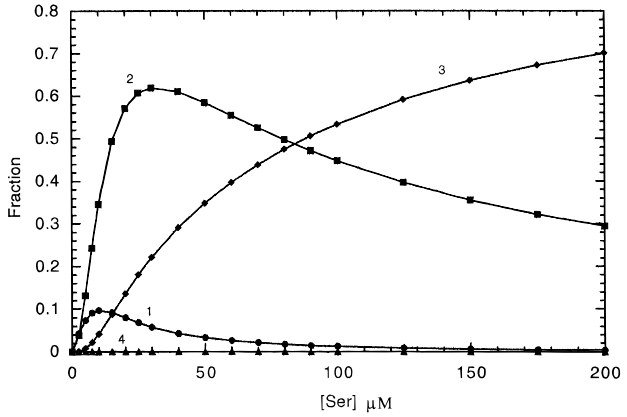

Fig. 5. Fractional distribution of species with bound serine. Fractional species distribution for enzyme with 1, ●; 2, ■; 3, ♦; and 4, ▲ serines bound.

molecular basis for this is unknown, but it may have something to do with the nature of the serine binding sites in that they are between two adjacent subunits rather than being contained entirely within a single subunit. Within this context, the negative cooperativity for the third and particularly for the fourth ligand might be explained on a physical basis. It has previously been suggested (Grant & Xu, 1998) that closure of a regulatory domain interface by one molecule of ligand could exclude binding by the second molecule of ligand at the same interface. The relatively low affinity for binding of the first ligand, and the higher affinity for the second ligand, is consistent with this hypothesis if the second ligand is binding at the opposite interface, and the third and fourth ligand then follow at both interfaces already containing a single ligand. This would suggest then that the positive cooperativity involves interactions at opposite interfaces that are ~110 Å apart. An order of binding can thus be envisioned where the binding of the first ligand initiates a conformational transition that allows the second ligand to bind with much higher affinity to the opposite interface. This is followed by the third ligand, which

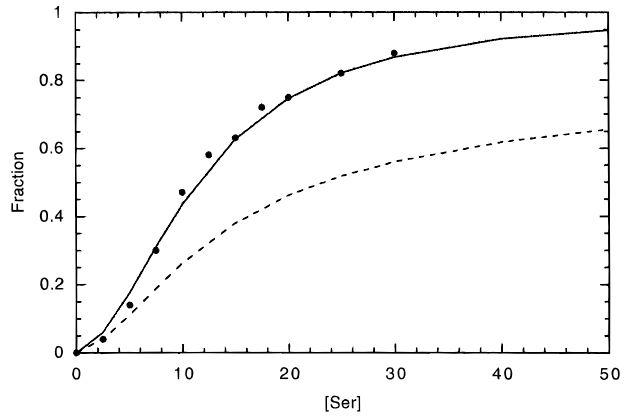

Fig. 6. Comparison of experimental and predicted inhibition patterns. Experimental serine inhibition data are plotted as symbols ●. The solid line represents the hypothetical inhibition curves predicted from the distribution in Figure 5 for $I \approx (0.5P_1 + P_2 + P_3 + P_4)$. The dashed line represents that for $I \approx (0.5P_1 + 0.5P_2 + P_3 + P_4)$.

binds with lesser affinity to one of the already occupied interfaces, and in so doing completes a global conformational transition that produces maximum inhibition of activity and an even lower affinity for the fourth ligand

Although the data support a sequential relationship between sites at opposite interfaces, they do not allow the determination of which serine binding sites are affecting which active sites. Nevertheless, although the question of the actual site linkage remains unanswered, this study clarifies the relationship between effector ligand binding and inhibition in PGDH, and presents a more detailed framework upon which the mechanism of allosteric regulation can be considered.

Materials and methods

PGDH was expressed, isolated, and assayed as previously described (Schuller et al., 1989; Al-Rabiee et al., 1996a). Activity was determined at constant temperature using α-ketoglutarate (Zhao & Winkler, 1996) as the substrate and by monitoring the decrease in absorbance of NADH at 340 nm (Tobey & Grant, 1986). Protein concentration was determined initially by the Bradford method as previously described (Bradford, 1976; Grant & Xu, 1998) and by quantitative amino acid analysis for the serine binding studies. All experiments are conducted with $PGDH_{4C/A}$, which is a form of the enzyme where the four native cysteine residues in each subunit have been converted to alanine. This construct has been described previously (Grant & Xu, 1998) and is used here for consistency of comparison to past studies. Kinetically, native PGDH and $PGDH_{4C/A}$ are very similar (Grant & Xu, 1998).

Equilibrium dialysis was performed in 500 μL dialysis cartridges obtained from Sialomed, Inc. (Columbia, Maryland). Dialysis was performed for 16 h with ^{3}[H]-L-serine as a tracer in appropriate concentrations of unlabeled L-serine. Cells were sampled in triplicate, and the average of 10 min counts were used to calculate concentrations of free and bound L-serine. The nominal PGDH concentration was 5 μM tetramer in all binding experiments. Amino acid analysis was used to determine the actual concentration and enzyme homogeneity was judged by SDS gels and specific activity.

Experimental data were fit to equations with the curve fitting program of Kaleidograph (Synergy Software, Reading, Pennsylvania). Coefficients of cooperativity and apparent dissociation constants for serine inhibition were determined by fitting the inhibition data to the Hill equation (Bell & Bell, 1988; Equations 1A & 1B):

$$Y = [L]^n/K_d + [L]^n \tag{1A}$$

$$\log(Y/1 - Y) = n\log[L] - \log K_d \tag{1B}$$

where Y is the fractional inhibition of the activity, L is the free L-serine concentration, and n is the coefficient of cooperativity.

Adair constants were determined by fitting the serine binding data to the Adair equation for a molecule with four binding sites (Bell & Bell, 1988; Equation 2).

$$Y = \frac{(L/K_1) + (2L^2/K_1K_2) + (3L^3/K_1K_2K_3) + (4L^4/K_1K_2K_3K_4)}{4(1 + (L/K_1) + (L^2/K_1K_2) + (L^3/K_1K_2K_3) + (L^4/K_1K_2K_3K_4))} \tag{2}$$

where Y is the fraction of sites occupied per total number of sites, L is the free L-serine concentration, and K_i are the stepwise Adair constants expressed as dissociation constants.

Intrinsic site dissociation constants were calculated from the Adair constants by using the following statistical relationships (Bell & Bell, 1988; Equations 3A–D):

$$K_1' = 4K_1 \tag{3A}$$

$$K_2' = 3K_2/2 \tag{3B}$$

$$K_3' = 2K_3/3 \tag{3C}$$

$$K_4' = K_4/4 \tag{3D}$$

where K_i' are the intrinsic dissociation constants.

The fractional distribution of bound protein species was computed from equations derived from the Adair equation and expressed as the fraction of protein tetramer with 1, 2, 3, or 4 serines bound per total protein tetramer (P_i/P_t) (Equations 4A–D).

$$P_1/P_t = \frac{L/K_1}{1+(L/K_1) + (L^2/K_1K_2) + (L^3/K_1K_2K_3) + (L^4/K_1K_2K_3K_4)} \tag{4A}$$

$$P_2/P_t = \frac{L^2/K_1K_2}{1 + (L/K_1) + (L^2/K_1K_2) + (L^3/K_1K_2K_3) + (L^4/K_1K_2K_3K_4)} \tag{4B}$$

$$P_3/P_t = \frac{L^3/K_1K_2K_3}{1 + (L/K_1) + (L^2/K_1K_2) + (L^3/K_1K_2K_3) + (L^4/K_1K_2K_3K_4)} \tag{4C}$$

$$P_4/P_t = \frac{L^4/K_1K_2K_3K_4}{1 + (L/K_1) + (L^2/K_1K_2) + (L^3/K_1K_2K_3) + (L^4/K_1K_2K_3K_4)} \tag{4D}$$

Acknowledgments

The authors wish to thank Professor Gary Ackers for his critical evaluation of the manuscript. This work was supported by grant #GM 56676 from the National Institutes of Health.

References

Al-Rabiee R, Lee, EJ, Grant GA. 1996a. The mechanism of velocity modulated allosteric regulation in D-3-phosphoglycerate dehydrogenase. Cross-linking adjacent regulatory domains with engineered disulfides mimics effector binding. *J Biol Chem 271*:13013–13017.

Al-Rabiee R, Zhang Y, Grant GA. 1996b. The mechanism of velocity modulated allosteric regulation in D-3-phosphoglycerate dehydrogenase. Site-directed mutagenesis of effector binding site residues. *J Biol Chem 271*:23235–23238.

Bell JE, Bell ET. 1988. *Proteins and enzymes*. Englewood Cliffs, New Jersey: Prentice-Hall, Inc. pp 465–470.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72*:248–254.

Grant GA, Kim SJ, Xu XL, Hu Z. 1999. The contribution of adjacent subunits to the active sites of D-3-phosphoglycerate dehydrogenase. *J Biol Chem 274*:5357–5361.

Grant GA, Schuller DJ, Banaszak LJ. 1996. A model for the regulation of D-3-phosphoglycerate dehydrogenase, a Vmax-type allosteric enzyme. *Protein Sci 5*:34–41.

Grant GA, Xu XL. 1998. Probing the regulatory domain interface of D-3-phosphoglycerate dehydrogenase with engineered tryptophan residues. *J Biol Chem 273*:22389–22394.

Schuller DJ, Fetter CH, Banaszak LJ, Grant GA. 1989. Enhanced expression of the *Escherichia coli* serA gene in a plasmid vector. Purification, crystallization, and preliminary X-ray data of D-3 phosphoglycerate dehydrogenase. *J Biol Chem 264*:2645–2648.

Schuller D, Grant GA, Banaszak L. 1995. The allosteric ligand site in the Vmax-type cooperative enzyme phosphoglycerate dehydrogenase. *Nat Struct Biol 2*:69–76.

Sugimoto E, Pizer LI. 1968a. The mechanism of end product inhibition of serine biosynthesis. II. Optical studies of phosphoglycerate dehydrogenase. *J Biol Chem 243*:2090–2098.

Sugimoto E, Pizer LI. 1968b. The mechanism of end product inhibition of serine biosynthesis. I. Purification and kinetics of phosphoglycerate dehydrogenase. *J Biol Chem 243*:2081–2089.

Tobey KL, Grant GA. 1986. The nucleotide sequence of the serA gene of *Escherichia coli* and the amino acid sequence of the encoded protein, D-3-phosphoglycerate dehydrogenase. *J Biol Chem 261*:12179–12183.

Zhao G, Winkler ME. 1996. A novel alpha-ketoglutarate reductase activity of the serA-encoded 3-phosphoglycerate dehydrogenase of *Escherichia coli* K-12 and its possible implications for human 2-hydroxyglutaric aciduria. *J Bacteriol 178*:232–239.