Hybrid Tetrarners Reveal Elements of Cooperativity in
Escherichia coli d-3-Phosphoglycerate Dehydrogenase*

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D-3-Phosphoglycerate dehydrogenase from Escherichia coli is a tetramer of identical subunits that is inhibited when L-serine binds at allosteric sites between subunits. Co-expression of two genes, the native gene containing a charge difference mutation and a gene containing a mutation that eliminates serine binding, produces hybrid tetrarners that can be separated by ion exchange chromatography. Activity in the hybrid tetramer with only a single intact serine binding site is inhibited by ~58% with a Hill coefficient of 1. Thus, interaction at a single regulatory domain interface does not, in itself, lead to the positive cooperativity of inhibition manifest in the native enzyme. Tetramers with only two intact serine binding sites purify as a mixture that displays a maximum inhibition level that is less than that of the native enzyme, suggesting the presence of a population of tetrarners that are unable to be fully inhibited. Differential analysis of this mixture supports the conclusion that it contains two forms of the tetramer. One form contains two intact serine binding sites at the same interface and is not fully inhibitible. The second form is a fully inhibitible population that has one serine binding site at each interface. Overall, the hybrid tetramers show that the positive cooperativity observed for serine binding is mediated across the nucleotide binding domain interface, and the negative cooperativity is mediated across the regulatory domain interface. That is, they reveal a pattern in which the binding of serine at one interface leads to negative cooperativity of binding of a subsequent serine at the same interface and positive cooperativity of binding of a subsequent serine to the opposite interface. This trend is propagated to subsequent binding sites in the tetramer such that the negative cooperativity that is originally manifest at one interface is decreased by subsequent binding of ligand at the opposite interface.

Escherichia coli d-3-phosphoglycerate dehydrogenase (PGDH, EC 1.1.1.95) catalyzes the first committed step in L-serine biosynthesis and is inhibited by the binding of L-serine to an allosteric site (1–3). PGDH is a tetramer of identical subunits and contains four catalytic sites as well as four effector sites (4). Each subunit is composed of three distinct domains, the substrate binding domain, the cofactor binding domain, and the serine binding or regulatory domain. Subunits make contact between adjacent regulatory domains at the regulatory domain interface (rdi) and between adjacent nucleotide binding domains at the nucleotide binding domain interface (ndi). The four catalytic sites are formed at the junction between the substrate and cofactor binding domains, and the four effector sites are formed at the two regulatory domain interfaces (Fig. 1). Hydrogen bonds to serine are contributed by both subunits at the regulatory domain interface, and serine appears to tether the two domains together through this hydrogen bond network (4, 5). Two distinctive cooperative processes appear to function in the inhibition of the enzyme by L-serine. First, the inhibition of activity in response to serine binding displays a sigmoidal behavior with a Hill coefficient of ~2. Secondly, serine binding itself displays characteristics of both positive and negative cooperativity (6). Furthermore, it has been demonstrated (7, 8) that these two processes can be uncoupled by specific amino acid residue mutations, producing mutant enzymes that have lost their cooperativity of inhibition while maintaining their cooperativity of serine binding. In addition, the degree of cooperativity in serine binding is affected by the binding of NADH (9) and the presence of phosphate ion (10) without an appreciable concomitant effect on the cooperativity of inhibition. These observations have led to the suggestion that the enzyme functions through distinct structural pathways that can be uncoupled by mutations or interactions with solvent constituents. Whereas these pathways may share structural elements, their uncoupling by mutation also demonstrates that they diverge structurally at some point. Thus, the interaction of these pathways and the interplay of specific catalytic and effector sites are of critical importance to understanding the regulatory mechanism.

Because PGDH is composed of four identical subunits, a mutation made in one subunit will occur symmetrically in all subunits. This property makes it difficult to separate intra-subunit effects from inter-subunit effects and to elucidate the precise relationship of one individual site to another. In order to address this, it is necessary to produce hetero-tetrarners where the sites are modified asymmetrically.

Similar studies utilizing hybrid oligomers have been reported for L-lactate dehydrogenase from Bifidobacterium longum (11, 12) and for porcine fructose 1,6-biphosphatase (13). Although these enzymes are homo-tetrarners like PGDH, they differ significantly in the arrangement of their substrate and effector binding sites. B. longum L-lactate dehydrogenase contains only two effector sites but has four active sites (14). The effector sites in L-lactate dehydrogenase are formed by residues from adjacent subunits, whereas the active sites are basically contained within each subunit. Fructose 1,6-biphosphatase
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Fig. 1. A diagram depicting the structure of the PGDH tetramer. Each identical subunit contains three distinct domains, the regulatory domain (r), the substrate binding domain (s), and the nucleotide binding domain (n). The active site is formed in a cleft between the substrate binding and nucleotide binding domains. The tetramers contact each other at interfaces between the regulatory domains and the nucleotide domains as depicted. L-Serine, the negative effector, binds in the regulatory domain interface.

contains four effector sites and four active sites (15). The substrate binding sites in fructose 1,6-bisphosphatase are at subunit interfaces, and the effector sites are contained within individual subunits. In PGDH, it is the effector sites that are found at subunit interfaces, and the substrate sites for PGDH interact with residues from adjacent subunits. In addition, the active site of PGDH is found in a distinct cleft between two subunit domains that is predicted to open and close during catalysis and remain closed in the inhibited state (4). Thus, there are distinct differences among these enzymes that could uniquely contribute to significant differences in their mode of action.

This study describes a method for producing and isolating specific hybrid tetramers of PGDH and analyzes the properties of PGDH tetramers containing zero, one, two, three, and four high-affinity serine binding sites.

MATERIALS AND METHODS

Protein was isolated and initially purified from bacteria utilizing 5′-AMP-Sepharose affinity chromatography as described previously (16, 17). Enzyme activity was determined by the change in absorbance at 340 nm due to the conversion of NADH to NAD⁺ at pH 7.5 (18) using α-ketoglutarate as the substrate (19).

All PGDH constructs used in this study are based on PGDH 4C, which is a form of PGDH in which the 4 cysteine residues in the PGDH subunit have been mutated to alanine residues. This construct behaves similarly to native PGDH and has been widely used in previous studies (5–10). The kₐ₁ and kₐ₂ for serine are identical to those of the native enzyme, and the Kᵣ for α-ketoglutarate is 5-fold higher than that of the native enzyme (16). It is chosen so that it can be used later for introduction of reporter groups at specific places on the subunit through new cysteine residues. For the sake of simplicity, this construct will be assumed as the background in all subsequent descriptions and will not be referred to specifically.

Construction of a Plasmid for the Co-expression of Two Distinct PGDH Genes—Hybrid or mixed tetramers were produced by expressing two individual PGDH genes on the same plasmid under control of a single promoter. The plasmid pTrc PGDH (16) was used to construct two intermediate plasmids, pTrc PGDH 1a and pTrc PGDH 1b (Fig. 2). pTrc PGDH 1a was made by introducing an XhoI site just after the stop codon and before the HindIII site in pTrc PGDH. This gene contained a charge difference mutation and a sequence tag as well as the native serine binding site residues. Charge variation was introduced into PGDH by the mutation of four surface glutamate residues (Glu-45, Glu-49, Glu-386, and Glu-387) to arginine residues. For the sake of simplicity, this construct will be assumed as the background in all subsequent descriptions and will not be referred to specifically.

pTrc PGDH

\[ \text{pTrc PGDH} \]

pTrc PGDH 1a

\[ \text{pTrc PGDH 1a} \]

pTrc PGDH 1b

\[ \text{pTrc PGDH 1b} \]

pTrc PGDH duo

\[ \text{pTrc PGDH duo} \]

residue. In this way, when hybrid tetramers are formed from this subunit (pTrc PGDH 1a) and a subunit containing the native valine residue (pTrc PGDH 1b), the exact ratio of subunits in the tetramer can be determined quantitatively by automated Edman sequencing. PTH-alanine and PTH-valine yield similarly (Val/Ala = 0.939 ± 0.002) in Edman sequencing so that integration of their peaks provides an accurate estimate of the subunit ratios. This construct is called 4ER/V4A, and the Kᵣ, kₐ₁, and IC₅₀ are the same for this construct as essentially indistinguishable from those of the unmutated enzyme.

pTrc PGDH 1b was made by introducing an XhoI site just before the NcoI site in pTrc PGDH. This gene contained the mutation of the serine binding site, N364A. Asn-364 is one of the three serine binding residues found in the regulatory domains of PGDH. This mutation decreases the IC₅₀ for serine from 10 μM in the native enzyme to 48 mM in the mutant (5). This effectively renders the site incapable of binding serine over the range of serine concentrations used in this study.

Mutations were produced in either pTrc PGDH 1a or pTrc PGDH 1b by PCR as described previously (16) in preparation for production of pTrc PGDH duo-αserine. pTrc PGDH duo-αserine was produced by placing the XhoI/HindIII fragment from pTrc PGDH 1b into the XhoI/HindIII sites in pTrc PGDH 1a. Simultaneous expression of both genes in pTrc PGDH duo-αserine was induced with isopropyl-1-thio-β-D-galactopyranoside and isolated with a 5′ AMP-Sepharose affinity column as described previously (17).

Purification of Charge-differentiated Hybrid Tetramers—Hybrid tetramers containing zero to four high-affinity L-serine binding sites were produced by expression of pTrc PGDH duo-αserine. Mutant hybrid tetramers were purified by chromatography on QAE-Sepharose in 1 mM potassium phosphate buffer, pH 7.5, 60 mM KSCN and eluted with a linear gradient of NaCl from 0 to 0.5 M. Fractions were pooled and dialyzed against appropriate buffers before further purification or analysis.

Pools that were not well resolved into homogeneous tetramers were re-chromatographed on QAE-Sepharose using the same conditions with a gradient from 0 to 0.3 M NaCl. The distribution of subunits within a pool was determined by automated Edman sequencing of the sequence tag region as described above.

Inhibition Analysis—Serine inhibition plots were fit to the Hill equation (20, 21),

\[ I = \frac{I_{\text{max}}[L]^{n}}{[I_{0.5}]^{n} + [L]^{n}} \]  

(Eq. 1)

where \( I_{\text{max}} \) is the maximum inhibition, \( I \) is the fractional inhibition, \( L \) is the concentration of ligand, \( n \) is the Hill coefficient, and \( I_{0.5} \) is the inhibitor concentration at half-maximal inhibition. Protein concentration was determined by quantitative amino acid analysis.

Serine Binding Analysis—Serine binding was measured by equilibrium dialysis in 200-μl dialysis chambers (Sialomed, Inc., Columbia, MD) purchased from the Nest Group (Southborough, MA). Dialysis was performed for 16 h with \( L^{-}[H] \)serine 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
t-serine. The nominal PGDH concentration was 5–10 μM tetramer in all binding experiments, and all binding was performed in the presence of 100 μM NADH. Serine binding data were fit to the Adair equation (21) for one (Eq. 2), two (Eq. 3), three (Eq. 4), or four (Eq. 5) sites using Kaleidograph (Synergy Software) as described previously (6, 7).

The Adair equations in the form of dissociation constants are shown below.

\[ Y = \left( \frac{1}{K_1} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} \]  
\[ Y = \left( \frac{1}{K_1} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} \]  
\[ Y = \left( \frac{1}{K_1} \right) + \left( \frac{2}{K_1^2} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} + \left( \frac{3}{K_1^3} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} \]  
\[ Y = \left( \frac{1}{K_1} \right) + \left( \frac{2}{K_1^2} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} + \left( \frac{3}{K_1^3} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} + \left( \frac{4}{K_1^4} \right) \frac{1}{1 + \left( \frac{L}{K_1} \right)} \]  

where \( Y \) is the fractional occupancy, \( L \) is the free ligand concentration, and \( K_i \) values are the stepwise Adair constants. Plots of \( Y \) versus free serine concentration were constructed with \( Y = r, Y = r/2, Y = r/3, \) and \( Y = r/4 \) for tetramers containing one, two, three, and four functional serine binding sites, respectively.

For the tetramer pool with two mutant sites, where three species are present (see Fig. 3), the Adair equation was modified as shown below to account for each species.

\[ Y = \left( \frac{1}{K_{1a}} \right) \]  
\[ Y = \left( \frac{1}{K_{1b}} \right) \]  
\[ Y = \left( \frac{1}{K_{1c}} \right) \]  
\[ Y = \left( \frac{1}{K_{1d}} \right) \]  

Intrinsic site dissociation constants were calculated from the Adair constants by using the following statistical relationships for a molecule where \( n \) are available and where \( K_i \) are the intrinsic dissociation constants (20).

\[ n = 2 \text{ sites; } K_{i} = 2 K_1 \]  
\[ n = 3 \text{ sites; } K_{i} = 3 K_1 \]  
\[ n = 4 \text{ sites; } K_{i} = 4 K_1 \]

RESULTS

Recombination of Co-expressed Subunits—The expression of pTrc PGDH duo/seryl is expected to produce a mixture of mutated hybrid tetramers as depicted in Fig. 3. Single species should be produced for hybrid tetramers containing zero, one, three, and four mutated serine binding sites. On the other hand, three species would be expected for the hybrid tetramer containing two mutated serine binding sites. However, because of the binding site symmetry and the fact that serine binds between subunits, two of these (the bottom two in the middle column in Fig. 3) are expected to be functionally equivalent because they contain one binding site at each interface. Unless otherwise noted, these two forms were treated equivalently.

Each group of tetramers will have different charge properties corresponding to the number of 4ER/V4A subunits they contain. The initial separation of the hybrid tetramers produced by the expression of pTrc PGDH duo/seryl is shown in Fig. 4. Peaks were pooled and analyzed by Edman degradation. Pools that were heterogeneous with respect to tetramer content as judged by the Val/Ala ratio were re-chromatographed as described under “Materials and Methods” (data not shown). The homogeneity of the final tetramer pools based on subunit distribution is shown in Table I. The kinetic parameters presented in Table II are similar for all hybrid tetramers, suggesting that the active sites are not compromised by the various mutations used.

Serine Inhibition of Hybrid Tetramer Activity—The serine inhibition profiles of the hybrid tetramers are shown in Fig. 5, and the results of fitting the data to the Hill equation are presented in Table III. The tetramer with four intact serine binding sites produced the expected inhibition pattern, which is similar to unmutated PGDH, where the Hill coefficient is ~2, and inhibition of activity is >95%. The tetramer with only three intact serine binding sites also displayed inhibition of activity of >95% but displayed a diminished sensitivity to serine at intermediate concentrations. The Hill coefficient was also reduced to a value of −1.6. The tetramer pool with only two


**TABLE I**


<table>
<thead>
<tr>
<th>Pool</th>
<th>Ala$^a$</th>
<th>Val$^a$</th>
<th>Fraction mutant subunit</th>
<th>Fraction native subunit</th>
<th>Mutant/native ratio</th>
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<tbody>
<tr>
<td>I</td>
<td>2.3</td>
<td>48.3</td>
<td>0.04</td>
<td>0.96</td>
<td>0/4</td>
</tr>
<tr>
<td>II</td>
<td>21.4</td>
<td>59.9</td>
<td>0.26</td>
<td>0.74</td>
<td>1/3</td>
</tr>
<tr>
<td>III</td>
<td>11.2</td>
<td>11.7</td>
<td>0.49</td>
<td>0.51</td>
<td>2/2</td>
</tr>
<tr>
<td>IV</td>
<td>25.0</td>
<td>3.3</td>
<td>0.75</td>
<td>0.25</td>
<td>3/1</td>
</tr>
<tr>
<td>V</td>
<td>22.6</td>
<td>0.9</td>
<td>0.96</td>
<td>0.04</td>
<td>4/0</td>
</tr>
</tbody>
</table>

* Each sample is taken from the pools shown in Fig. 3. Some pools may have been re-chromatographed for better separation. No attempt was made to load identical quantities into the sequencer reaction cell. Values represent the peak integration from the PTH-amino acid chromatogram at the appropriate cycle.

**TABLE II**


<table>
<thead>
<tr>
<th>Mutant subunits/tetramer</th>
<th>Functional effector sites/tetramer</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>max (\mu\text{mol} \text{min}^{-1} \text{mg}^{-1})</td>
<td>(s^{-1})</td>
<td>(s^{-1} \mu\text{mol}^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>0.41 ± 0.15</td>
<td>3.7 ± 0.5</td>
<td>11.0</td>
<td>2.7 × 10$^4$</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>0.60 ± 0.06</td>
<td>5.0 ± 0.2</td>
<td>14.9</td>
<td>2.5 × 10$^4$</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.41 ± 0.06</td>
<td>3.4 ± 0.2</td>
<td>10.1</td>
<td>2.5 × 10$^4$</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.42 ± 0.08</td>
<td>3.7 ± 0.2</td>
<td>11.0</td>
<td>2.6 × 10$^4$</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.46 ± 0.10</td>
<td>3.9 ± 0.3</td>
<td>14.6</td>
<td>3.2 × 10$^4$</td>
</tr>
</tbody>
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**DISCUSSION**

A complete set of hetero-tetramers of PGDH have been produced and isolated that differ sequentially in the number of functional serine binding sites that they contain. This has been accomplished by co-expression of two genes for PGDH in *E. coli*. One gene contained the single serine binding site mutation, and the other gene contained the native serine binding site as well as charge mutants on the surface of the subunit and a sequence tag at the amino terminus of the polypeptide chain. Upon expression and folding, the subunits combined to give the expected mixture of tetramers, which were then separated on an ion exchange column by virtue of the number of charge-mutated subunits each tetramer contained. The stoichiometry of the hetero-tetramers was verified by automated Edman sequencing of the sequence tag region.

The serine inhibition profiles of the hybrid tetramers display distinctly different patterns. The activity of the tetramer containing only a single intact serine binding site (three mutated sites) is inhibited to only ~58%. The simplest explanation for this observation is that the binding of a single effector molecule at one rdi can effectively inhibit both active sites in the subunits that contribute to that interface. The observation that the extent of inhibition is slightly greater than 50% also suggests that there may be a small influence on the activity of the intact serine binding sites produced a Hill coefficient of ~1.2 but showed even more reduction in sensitivity to serine and a reduced total inhibition of activity of ~85–90%. The tetramer with only a single serine binding site displayed a Hill coefficient of 1, but its activity is inhibited only ~58%. As expected, the tetramer with no intact serine binding sites was not inhibited by serine.

Serine Binding to the Hybrid Tetramers—The serine binding curves fitted to the appropriate Adair equations are presented in Fig. 6, and the derived dissociation constants are presented in Table IV. Note that all serine binding experiments were performed in the presence of saturating NADH, which moderates the degree of cooperativity (9). The stoichiometry of serine binding measured for these mutants correlates well with the expected number of intact serine binding sites. The data for tetramers with one, three, and four intact serine binding sites can be fit satisfactorily with Adair equations derived for the respective number of sites. A special case exists for the tetramer with only two intact serine binding sites. As shown in Fig. 3, three species are predicted statistically, and based on symmetry, at least two of these are expected to be functionally equivalent. Attempts to fit the data to a single Adair equation for two sites failed to produce a satisfactory fit. However, when the fit was attempted for an Adair equation composed of three successive two-site equations with equal weight, the fit shown in Fig. 6 was achieved. This fit produced binding parameters (shown in Table IV as three separate entries) that indicated that two of the three predicted species were equivalent. This corresponds to the predicted functional equivalency of two of the three species based on symmetry considerations.
catalytic sites in the other two subunits as well. This presumably results from a conformational change induced by the binding of the first serine ligand. The Hill coefficient of this tetramer is 1, which also suggests that interaction across this regulatory domain interface does not, in itself, produce the cooperativity of inhibition of catalytic activity seen in the native enzyme. However, this same interaction undoubtedly contributes to cooperativity of binding of subsequent ligands in the native enzyme. This interpretation is also consistent with the observation that all of the other hybrid tetramer pools, all of which contain tetramers that bind serine at both regulatory domain interfaces, display positive cooperativity for serine inhibition. Because this tetramer contains only a single serine binding site, no cooperativity of serine binding is possible, and

![Fig. 6. Plots of serine binding data. Left panel, plots of the number of mols of L-serine (r) bound per tetramer versus the free serine concentration determined by equilibrium dialysis. The symbols are the experimental data, and the solid lines are the fit of the data to the appropriate Adair equation as indicated in the text. Results of PGDH hybrid tetramers with four (■), three (●), two (○), and one (▲) high-affinity serine binding site(s) are shown. Right panel, Scatchard plots of the data shown on the left.](image)

<table>
<thead>
<tr>
<th>Mutant subunits/tetramer</th>
<th>Functional effector sites/tetramer</th>
<th>n</th>
<th>$S_{0.5}$</th>
<th>$I_{max}$</th>
<th>$X^2$</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td></td>
<td>2.16 ± 0.11</td>
<td>1.5 ± 0.1</td>
<td>0.98 ± 0.01</td>
<td>0.020649</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td></td>
<td>1.60 ± 0.17</td>
<td>4.3 ± 0.3</td>
<td>0.96 ± 0.02</td>
<td>0.024799</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td></td>
<td>1.20 ± 0.11</td>
<td>7.6 ± 0.8</td>
<td>0.89 ± 0.03</td>
<td>0.015707</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td></td>
<td>1.03 ± 0.06</td>
<td>7.7 ± 0.5</td>
<td>0.58 ± 0.01</td>
<td>0.001492</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td></td>
<td>No inhibition observed</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\text{TABLE III}$

Serine inhibition parameters

Parameters were derived from fitting the data to the Hill equation. $n$ is the Hill coefficient, $S_{0.5}$ is the serine concentration at one-half the maximum inhibition level, $I_{max}$ is the maximum inhibition level, $X^2$ represents the cumulative deviation between the data and the fit, and $R$ is the correlation coefficient.

$\text{TABLE IV}$

Serine binding parameters

Binding data are expressed as dissociation constants (μM). Adair constants are denoted as $K_i$ and intrinsic binding constants as $K_i'$. | Mutated sites | 0 | 1 | 2 | 3 | 4 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$</td>
<td>3.0 ± 1.4</td>
<td>6.2 ± 2.5</td>
<td>20.0 ± 0.0003</td>
<td>8.3 ± 0.0007</td>
<td>8.3 ± 0.0007</td>
</tr>
<tr>
<td>$K_2$</td>
<td>3.5 ± 2.0</td>
<td>3.8 ± 1.4</td>
<td>VL</td>
<td>6.8 ± 0.0009</td>
<td>6.8 ± 0.0009</td>
</tr>
<tr>
<td>$K_3$</td>
<td>13.6 ± 6.6</td>
<td>112 ± 23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_4$</td>
<td>18.7 ± 5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$K_1'$</td>
<td>12</td>
<td>19</td>
<td>40</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>$K_2'$</td>
<td>5</td>
<td>4</td>
<td>VL</td>
<td>3</td>
<td>3</td>
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<tr>
<td>$K_3'$</td>
<td>9</td>
<td>37</td>
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</tr>
<tr>
<td>$K_4'$</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X^2$</td>
<td>0.00788</td>
<td>0.02353</td>
<td>0.02204</td>
<td>0.06257</td>
<td></td>
</tr>
<tr>
<td>$R$</td>
<td>0.99748</td>
<td>0.98957</td>
<td>0.99498</td>
<td>0.97159</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Mutant sites per hybrid tetramer.
$^b$ Derived from an Adair equation summing three components with two constants each.
$^c$ VL, very large i.e. >10^4.
$^d$ Values for intrinsic constants are determined from the Adair constants by the statistical relationships presented under "Materials and Methods" and rounded to the nearest integer.

Fitting statistics: $R$ is the correlation coefficient, and $X^2$ is the sum of the residuals.
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FIG. 7. Fitting the serine inhibition data for the two-site species to two successive Hill equations. The symbols (●) are the data from Fig. 5, and the fit is produced with two successive Hill equations weighted in a one-third to a two-thirds ratio as predicted in Fig. 3. The $X^2$ value for the fit is 0.017265, and the correlation constant, $R$, is 0.99489. The dashed line indicates the hyperbolic response to the dual Hill equation (i.e., $n = 1$).

The data produce a hyperbolic binding curve yielding a single dissociation constant.

The results for the tetramer species containing two and three intact binding sites are particularly revealing. The pool containing tetramers with only two intact serine binding sites (two mutant sites) displays a maximum level of inhibition of activity that is less than that of native enzyme. This suggests the presence of a population of tetramers whose activity is unable to be fully inhibited. Indeed, the distribution of mutant tetramers shown in Fig. 3 predicts that one-third of the tetramers will bind serine at only one regulatory domain interface. This species would be expected to behave similarly to the tetramer with only a single serine binding site discussed above because serine interaction occurs only at one interface. That is, its activity should be $-58\%$ inhibited at maximum. Thus, if the activity of one-third of the species is inhibited to $-58\%$ of total, and that of two-thirds of the species is inhibited $-98\%$, the catalytic activity of the whole population would be inhibited by $-85\%$, which is what is seen experimentally.

Fitting the binding data to three successive Adair equations for two binding sites each produces a fit that partitions the species in a one-third and two-thirds distribution of total as predicted. The species predicted to bind serine at both regulatory domain interfaces shows positive cooperativity for the second ligand. The species predicted to have both intact serine binding sites at the same rdi shows that two-third of the species will bind serine at only one regulatory domain interface.

Fitting the serine inhibition profile of the pool with two intact serine binding sites to two successive Hill equations weighted for the predicted distribution of these species produces a fit (Fig. 7) in which the smaller population (one-third) displays a Hill coefficient of $<1 (0.67 \pm 0.42)$ and an $I_{0.5}$ of 45 μM, and the larger population (two-thirds) displays a Hill coefficient of $>1 (1.4 \pm 0.18)$ and an $I_{0.5}$ of 6 μM. The $I_{0.5}$ for this smaller population is quite close to the $K_d (40 \mu M)$ determined for serine binding of the tetramer with one intact serine binding site (see Table IV). The larger population has a Hill coefficient indicating positive cooperativity and an $S_{0.5}$ within the range for the $K_d$ values determined for this population from serine binding. Thus, both the multicomponent Adair equation and the multicomponent Hill equation produce fits that indicate that the population at one-third of the total species basically mimics the characteristics of the tetramer with only a single intact serine binding site.

The tetramer with three intact serine binding sites (one mutated site) has a Hill coefficient of 1.6 and displays nearly complete inhibition at higher serine concentrations. The binding parameters show the expected positive cooperativity for the second ligand and negative cooperativity for the third ligand. However, note that the negative cooperativity for the third ligand, which binds at the same interface as the first ligand, is not as extreme as that seen for the tetramer with two intact serine sites at the same interface discussed above. This suggests that the binding of the second ligand at the interface opposite to that where the first ligand binds is exerting its own positively cooperative effect on the opposite interface. Although the dissociation constant for the third ligand is higher than that for the first two, indicating negative cooperativity, it is significantly lower than if it were not being influenced by an interaction at the opposite regulatory domain interface. This apparent lessening of negative cooperativity is seen to an even greater degree for the unmutated tetramer in which all four sites interact with serine.

Taken together, these results reveal a pattern in which binding of serine to a second site at the same interface is negatively cooperative, and binding of serine to a second site on the opposite interface is positively cooperative. Fig. 8 depicts this concept in diagrammatic form. The positive cooperativity observed for serine binding is mediated across the rdi and is a result of a conformational change induced at the second interface that increases the binding affinity for ligand at that interface.
the same time, binding at a particular interface is negatively cooperative for additional ligand binding at that interface but can be modified by interaction at the opposite interface. In terms of the nomenclature used in Fig. 8, binding at site 1 enhances affinity at site 2 but reduces affinity at site 3. However, subsequent binding at site 2 moderates the negative cooperativity at site 3 to the extent that measurable ligand binding takes place. Similarly, binding to site 3 ameliorates the negative effect that occupancy at site 2 exerts on site 4 so that measurable binding also occurs at site 4.

Overall, the binding of a single serine at each interface produces substantial inhibition of all four active sites in a positively cooperative manner. This appears to be the major factor in the regulation of the enzyme. Binding of additional serines has a lesser effect but does incrementally increase the degree of inhibition obtained and incrementally decreases the subsequent dissociation constants (higher affinity). However, because two regulatory domain interfaces regulate four active sites, the binding of a second serine at each interface may not play a significant role in the regulation of the enzyme.

The picture of PGDH that is emerging from these and previous studies suggests that there is a significant entropic element to the regulation of this enzyme. PGDH has anecdotally been inferred to be a very flexible tetramer because of the large degree of freedom experienced at one regulatory domain interface translates to the opposite regulatory domain interface as a result of producing a more rigid tetramer overall. The increasing rigidity results in an inability of the enzyme to turn over rather than a decrease in the ability of substrates to bind, consistent with the observation that it is largely a “V”-type enzyme (2).

This investigation has allowed the determination of the order of binding of effector molecules to PGDH and provided evidence to discern the characteristics of individual effector binding sites and how they directly effect the characteristics of the other binding sites and the active sites. Additional investigations with hybrid tetramers containing other mutations will continue to define the site to site relationship in this enzyme as well as the structural elements that mediate the process.

REFERENCES