The Mechanism of Velocity Modulated Allosteric Regulation in D-3-Phosphoglycerate Dehydrogenase

CROSS-LINKING ADJACENT REGULATORY DOMAINS WITH ENGINEERED DISULFIDES MIMICS EFFECTOR BINDING*

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D-3-Phosphoglycerate dehydrogenase (PGDH) (EC 1.1.1.95) from Escherichia coli is an allosterically regulated enzyme of the V_{\text{max}} type. It is a tetramer of identical subunits and each subunit is made up of three identifiable domains, the cofactor binding domain, the substrate binding domain, and the regulatory domain. Each subunit contacts two other subunits through adjacent cofactor binding domains and through adjacent regulatory domains. L-Serine, the physiological effector, inhibits catalytic activity by apparently tethering regulatory domains from adjacent subunits together through the formation of hydrogen bonds to each subunit. This investigation demonstrates that cross-linking adjacent regulatory domains with engineered disulfides produces catalytic inhibition in the absence of inhibitor in a manner similar to that produced by the inhibitor. The inhibition due to cross-linking can be completely reversed in a concentration dependent manner by dithiothreitol. The active mutant enzyme, containing the engineered cysteines in the reduced state, retains its ability to be inhibited by L-serine, although at a 100-fold higher concentration. Hill plots of the serine inhibition of mutant and native enzyme indicate that the number of interacting sites remains at 2 in the mutant enzyme. The reversible inhibition of enzyme activity that results from tethering adjacent regulatory domains with engineered disulfides suggests that these domains move in some manner relative to one another during the active to inhibited state transition. These observations support the model which predicts that catalytic activity is regulated by the movement of rigid domains about flexible hinges and that effector binding prevents this by locking the regulatory domains in a state that produces an open active site cleft.

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The abbreviations used are: PGDH, D-3-phosphoglycerate dehydrogenase; DTT, dithiothreitol; IPTG, isopropyl-\(\beta\)-\(D\)-galactopyranoside; PCR, polymerase chain reaction.
association and dissociation of effector. The model predicts that relaxation of subunit association about this hinge, which would be the case when the effector is not bound, releases the substrate binding domain from its contacts with the regulatory domain so that it is now free to close the active site cleft for catalysis to occur. This latter hinge has been referred to as a "piano hinge" because it may act along a relatively long axis at the regulatory domain interface. The motion of the piano hinge is postulated to be directly modulated by binding of the effector molecule and functions at the point where two regulatory domains contact each other. This article demonstrates that, as predicted by the model, cross-linking the regulatory domains from adjacent subunits with engineered disulfides appears to prevent this motion and mimics effector binding, resulting in the reversible modulation of enzyme activity.

MATERIALS AND METHODS

Restriction enzymes were from Boehringer Mannheim or New England Biolabs. T4 DNA ligase was from Boehringer Mannheim. pBlue-script SK + was from Stratagene. pTRc99A and 5'-AMP-Sepharose were from Pharmacia. All PCR reagents were from Perkin-Elmer. Klenow fragment was from Promega. Agarose, Sepharose S-200 HR, gel filtration calibration proteins, IPTG, ampicillin, polyethylenimine, imidazole, l-cysteine, and NADH were from Sigma. Ellman's reagent (5,5-dithio-bis(2-nitrobenzoic acid)) was from Pierce.

DNA sequencing was performed with the Sequenase version 2.0 sequencing kit from U.S. Biochemical Corp. Restriction fragments were isolated from agarose gels with the Gene Clean kit from BIO 101 Inc.

The serA gene encoding PGDH was derived from pSAWT (11) and placed in the expression vector pTRc99A for expression by IPTG induction using the Ncol and HindII sites that flank the serA gene. This construct was named pTRcPGDH. Mutants were produced by PCR mutagenesis using standard procedures (13). In preparation for PCR mutagenesis, the serA gene was placed into pBlue-script SK +, in which an Ncol site had been added between the BamII and PstI sites of the multiple cloning site. The gene was initially cut from pSAWT with Ncol and HindII and spliced into the altered pBlue-script SK + at these same sites. Subsequently, the linear template for PCR was produced from this construct with an Ncol and Sall digest. The area to be mutated was between internal EcoRI and HindII sites that were used for the outside PCR primers and for insertion of the PCR product back into pTRcPGDH. All mutations were confirmed by Sanger dideoxy sequencing.

PGDH was expressed in E. coli following IPTG induction and isolated as described previously (11). Oxidation during purification could be reduced if all buffers contained 10 mM DTT with the exception of the final buffer used to elute the enzyme from the affinity column. After elution from the affinity column, a short dialysis (2-4 h) against elution buffer (2-4 liters) can be used to remove residual DTT if necessary. The enzyme was assayed at 25°C by following the decrease in absorbance at 340 nm in the presence of saturating levels of NADH and hydroxyproprionic acid phosphate as substrate (12). One unit of activity corresponds to the conversion of 1 nmol of NADH to NAD”/min. Protein concentration

![Image of Tetrameric structure of native PGDH and the regulatory domain interface.](http://www.jbc.org/)

Fig. 1. Tetrameric structure of native PGDH and the regulatory domain interface. Panel A, the subunits of the tetramer of PGDH are shown in alternating blue and red. Within each tetramer the
was determined by absorbance at 280 nm using an $E_{1\text{cm}}^{\text{1%}}$ = 6.7. Oxidation was performed by exposing the enzyme solution to air at 4°C. Reduction was performed at 25°C by adding the specified level of dithothreitol. Titration with Ellman’s reagent was performed according to published procedures (14) using l-cysteine to generate a standard curve. SDS-PAGE was performed in the absence of reducing agent according to the method of Laemmli (15). Gel filtration chromatography with Sepharose S-200 HR was performed in a 1.6 × 110-cm column with a flow rate of 0.4 ml/min in 20 mM imidazole buffer, pH 6.2, 1 mM EDTA. The column was calibrated with blue dextran, β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000).

The structure of PGDH was viewed with a Silicon Graphics IRIS (SGI) molecular graphics system Using SYBYL (Tripos Inc.) software. The SYBYL BIOPOLYMER program was used to simulate mutagenesis.

RESULTS

Rationale for Cross-link Location—If the close association of regulatory domains and closure of the regulatory domain interface is at the heart of the regulatory mechanism of PGDH as the model predicts, then tethering them together in a covalent fashion would be predicted to produce catalytic inhibition in a manner similar to that seen when L-serine is present. Thus, placing cysteine residues on each subunit at the domain interface so disulfide bridges can be made connecting the two subunits should cause the body of the domains to remain fixed relative to one another and thus prevent movement about the “hinge.” The use of a disulfide bridge to accomplish this also allows the tether to be susceptible to reduction and thus produce a reversible effect. Moreover, the ability to reverse the cross-link with a reducing agent and restore activity would provide evidence that the rest of the protein is not compromised as a result of the mutation or the oxidation process.

Inspection of the structure of PGDH indicated that residues Ala359 and Gly349 are appropriately located at the regulatory domain interface (Fig. 1B) at the solvent exposed surface of the tetramer and have their α carbons pointing at each other in the backbone structure. Because of the 180° asymmetry at the interface, two residues in each domain must be mutated so that the resultant cysteines are adjacent to one another across the interface. Mutagenesis simulation indicates that mutating these residues to cysteine would place their β carbons within 3.9 Å of each other, well within the distance needed for disulfide formation. Finally, within each domain, Ala359 and Gly349 are separated by approximately 15 Å so that intra-domain disulfides cannot form. They are also more than 20 Å from any of the native cysteines in PGDH.

Oxidation of A359C,G349C PGDH—When the A359C,G349C PGDH mutant was exposed to air, a gradual loss of activity was observed (Fig. 2). Native enzyme under the same conditions retained nearly 100% activity over the same time period. Table I compares the specific activity of native and mutant PGDH with respect to time and oxidation state. Full activity can be restored at any time by treatment with DTT and the rate of recovery of activity is dependent on the concentration of DTT. The activity of the native enzyme is not affected by DTT treatment. These results have been repeated four times with four independent enzyme preparations with no significant variability observed. Moreover, oxidative inhibition of mutant enzyme can be reversed on enzyme that has already been oxidized by reducing it with DTT and then removing the DTT by gel filtration or rapid dialysis.

Titration of oxidized, inhibited A359C,G349C PGDH with Ellman's reagent indicates that approximately 1.9 sulfhydryl groups are oxidized per subunit when the enzyme is 90% inhibited (Table II). This compares very well with the expected value of 2 sulfhydryl groups/subunit for completely cross-linked enzyme due to the engineered cysteine mutations. Analysis with Ellman’s reagent at low levels of inhibition indicates approximately 6 cysteine residues/subunit, as expected.

Effect of Oxidation and Reduction on Subunit Association—Molecular weight analyses were performed on native and A359C,G349C PGDH using gel filtration (nondenaturing) and SDS gels (denaturing) to assess the nature of the subunit cross-linking as a result of disulfide formation. These data are presented in Table III and Fig. 3.

If cross-linking due to disulfide formation is taking place across the interface between two regulatory domains as intended, analysis under denaturing conditions, in the absence of reducing agent, should produce a monomeric species for oxidized native enzyme and a dimeric species for the oxidized mutant. The dimer would result because two subunits would be covalently linked at the point where their respective regulatory domains contact each other. Furthermore, if cross-linking was


**TABLE III**

Molecular weight analysis of oxidized native and A359C,G349C PGDH

|                  | SDS Gel filtration |
|------------------|--------------------|
| Native PGDH      | 44,000*            |
| A359C,G349C PGDH | 88,000             |

*A The molecular weight of a PGDH subunit is approximately 44,000 so that a dimer would be approximately 88,000 and the tetramer would be approximately 176,000.

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**DISCUSSION**

The engineered cysteines were placed at specific sites in PGDH based on detailed knowledge of the structure and the model of allosteric inhibition. Only the mutated enzyme is inhibited under oxidative conditions and then activated in response to a mild reductant well known to reduce disulfide bonds. The native enzyme does not respond in this way and is not affected by DTT. The mutant enzyme, when fully reduced, shows catalytic competence equal to the native enzyme. Therefore, the observed result is specifically a consequence of the mutation and the complete retention of specific activity in the reduced form indicates no significant changes have occurred in enzyme structure or catalytic mechanism. Furthermore, Hill plots of the serine inhibition of the native and mutated enzyme, show that the Hill coefficient remains unchanged. Thus, although the concentration at which serine affects the mutant form is higher, the mutant form retains 2 interactive sites.

The SDS gel data clearly show that an intersubunit cross-link is forming and the native gel filtration data show that cross-links are not forming from tetramer to tetramer. Therefore, the intersubunit cross-link is within each tetramer. The crystal structure shows that the only cysteines within the tetramer that are close enough to each other to form intersubunit cross-links are the ones that were engineered into the molecule. All of the native cysteines are greater than 20 Å from each other across subunits. The crystal structure of PGDH indicates that disulfides do not exist in the native form. Moreover, inhibition with serine is completely reversible with removal of serine and does not depend on the redox state of the solution. There are only 2 native cysteines in PGDH that are in close enough proximity that an intrasubunit disulfide could be formed. Mutating these two native cysteines to alanine produces an enzyme that is indistinguishable from the native form in activity and its ability to be inhibited by serine. Thus, these cysteines are not operative in the native mechanism. Furthermore, titration of free thiols in A359C,G349C PGDH shows that approximately 1.9 cysteine residues are oxidized when the enzyme is 90% inhibited and that the unoxidized enzyme contains the expected 6 cysteines/subunit. Thus, these data are entirely consistent with oxidative inhibition of PGDH being due to the engineered cysteine residues at the regulatory domain interface.

These data indicate that tethering the regulatory domains from adjacent subunits of PGDH across their interface results in a reversible inhibition of PGDH in the absence of added natural inhibitor. Since the tetrameric nature of the enzyme has been shown not to change in response to effector mediated inhibition or domain cross-linking, this suggests that the regulatory mechanism involves the movement of one regulatory domain relative to the other. The effect of disulfide cross-linking...
linking is to convert a dynamic situation into a static situation where the domains are essentially locked in place.

However, the exact nature of the motion of adjacent regulatory domains relative to one another remains to be determined. Inspection of the structure shows that the residues that appear to bind the effector, l-serine, are located at the turns that connect the α-helix nearest the interface with the β sheet strands (see Fig. 1). This places the binding site for l-serine above the plane of the β sheet and below the helices. It is tempting to speculate that perhaps the hinging motion involves the movement of the α-helices at the interface away from each other with the junction of the β strands in the sheet acting as some sort of fulcrum. However, this view would require flexibility in the β sheet at the interfacial junction, a phenomena for which there is very little precedent. An alternative view might entail movement of the α-helices independently of the β sheet, with the hinge region being limited to the connecting turn that contains the effector binding residues. Effector binding to this region may stabilize the hinge. This, however, would seem to require movement of the inner helices relative to the outer helices, which seems unlikely because of the proximity of adjacent helices.

While the exact nature of regulatory domain interaction and the location of the hinge remain to be elucidated, these results support the main feature of the proposed model for the allosteric mechanism. That is, catalytic inhibition results when the regulatory domains are tethered to each other by the effector molecule, l-serine, and these domains must move in some way relative to one another during the active to inhibited state transition.

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