The mode of binding of NADPH and oxidized glutathione to the flavoenzyme glutathione reductase has been determined by x-ray crystallography. Furthermore, two intermediates of the reaction have been produced in the crystal and have been structurally elucidated. All these analyses were done at 0.3 nm resolution. The NADPH to the substrate GSSG via flavin and the redoxactive protein disulfide bridge. This is consistent with the scheme that has been postulated from biochemical, spectroscopic, and model studies. The drawing contains the two dinucleotides FAD and NADPH, the substrate GSSG, as well as several amino acid residues which are considered important for catalysis. The positions of the residues and of FAD have been fitted to its respective map using the interactive display system at Uppsala (28). For placing GSSG we used our own interactive display system.

RESULTS

The exact geometry of the ligands bound to glutathione reductase is given in Fig. 1. The drawing contains the two dinucleotides FAD and NADPH, the substrate GSSG, as well as several amino acid residues which are considered important for catalysis. The positions of the residues and of FAD have been derived from a multi-isomorphous replacement map of 0.2 nm resolution (24). NADPH and GSSG were fitted to difference electron density maps based on Soaks A and B (Table I), respectively. Soak B contained NADP+ in addition to the substrate GSSG in a 2:1 ratio. The results of these experiments have been described earlier (24, 27). The binding sites of the substrates were derived from difference-Fourier maps. NADPH and GSSG were fitted to an electron density map of glutathione reductase (24).

MATERIALS AND METHODS

Purified glutathione reductase (20) was a gift from Dr. R. H. Schirmer (University of Heidelberg, West Germany). The crystallization of the enzyme has been described earlier (25). Crystals of Form B were transferred to standard storage solutions (26) containing the dinucleotides FAD and NADPH, the substrate GSSG, as well as several amino acid residues which are considered important for catalysis. The positions of the residues and of FAD have been fitted to its respective map using the interactive display system at Uppsala (28). For placing GSSG we used our own interactive display system.

RESULTS

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The enzyme uses NADPH as a source of reduced glutathione (EC 1.6.4.2) is a ubiquitous FAD-containing enzyme (1). It catalyzes the reaction

\[ H^+ + NADPH + E = NADP^+ + E_H \]

where the intermediate \( E_H \) is the stable 2-electron reduced form of the enzyme. The function of the enzyme is to keep the cellular concentration of the reduced form of glutathione (GSH) high and that of its oxidized form (GSSG) low. Recent publications report a value of about 300 for the ratio [GSH] /[GSSG] (2). The enzyme uses NADPH as a source of reduction equivalents. Its substrate GSSG as well as its product GSH are important for a broad range of cellular functions, e.g., cell division (3, 4), amino acid transport through membranes (5), regulation of enzymatic activity (6, 7), damage repair (8, 9), drug metabolism (10), and detoxication (11). Moreover, the enzyme has been identified as one of the target molecules of the widely applied antitumor drug carmustine (12, 13).

Glutathione reductase is a member of the family of disulfide reductases. This family also includes lipoamide dehydrogenase and thioredoxin reductase, both FAD-containing redox enzymes that interact with disulfide substrates (1, 14). Lipoamide dehydrogenase especially is very similar to glutathione reductase, both in structural and mechanistic aspects (15, 16). Also the bacterial enzyme mercuric reductase resembles glutathione reductase (17).

In our experiments we used glutathione reductase from human erythrocytes, an enzyme of EC 1.6.4.2 that has been fitted to an electron density map of 0.2 nm resolution, yielding the complete structure of the molecule (24). Here we report on the crystal structures of the complexes between glutathione reductase and its reaction partners NADPH and GSSG as well as on two reaction intermediates and a covalently modified enzyme. The observed geometries of these complexes and the intermediates allow some conclusions on the mechanism of action of this flavoenzyme.
to GSSG. Here the difference density revealed that only the adenosine moiety of oxidized NADP⁺ is bound to the oxidized enzyme, E, whereas from the pyrophosphate onward the nicotinamide moiety is dangling in the solvent.

The intermediate state EH₂ in the catalytic cycle of the enzyme was produced in Soak C. The resulting difference electron density showed one major change as compared to the oxidized enzyme; the disulfide bridge Cys-58-Cys-63 has opened and the sulfur of Cys-58 has moved by about 0.1 nm toward the sulfur of glutathione I. In Fig. 1 these two sulfurs are marked by dots. The opening of the disulfide bridge Cys-58-Cys-63 has also been observed in Soak A, where NADPH had reduced the enzyme to its EH₂ form.

The sulfhydryl of Cys-58 created in this reduction is a highly reactive nucleophile and can be rather selectively modified with 2-iodoacetamide to give EHR, the blocked 2-electron reduced enzyme. This reaction could be demonstrated for the yeast (29) and the human enzyme (22). In the human enzyme the reaction occurs when NADPH (or dithioerythritol) and 2-iodoacetamide are applied at the same time (22). When we tried a similar procedure on the crystalline human enzyme in Soak D (Table I), the resulting difference-Fourier map showed one significant positive peak with size and shape corresponding to a carbonated methyl group plus the displaced sulfur of Cys-58. EHR molecules are no longer able to react with glutathione, or to transfer electrons via FAD to NADP⁺ in the back reaction. Therefore, crystals of EHR are stable in aerobic buffers. So far, we have kept some of them for more than 1 year without any indications of reoxidation.

In two further experiments, Soaks E and F (Table I), we obtained the mixed disulfide between glutathione I and Cys-58 of the protein. The two difference electron density maps were essentially identical with each other. Glutathione I was bound like the glutathione I moiety of GSSG in Soak B, except for the disulfide bridge which now is glutathione I-Cys-58 instead of glutathione I-glutathione II. The reoxidative disulfide bridge between Cys-58 and Cys-63 is open. The position of glutathione II is occupied at a level of about 20% of glutathione I.

In order to illustrate the gross arrangement of ligands in glutathione reductase we give a structural overview in Fig. 2. The enzyme consists of two subunits, which are related to each other by a 2-fold axis. The subunits are covalently connected by a single disulfide bond across this molecular axis. The 18 NH₂-terminal residues have no defined structure in the crystal. The functional role of this flexible arm is not known. The arm contains a cysteine at position 2. Considering the gross geometry of the enzyme Cys-2 could possibly reach Cys-58 of the reoxidative disulfide bridge. The remaining 460 residues of the polypeptide chain are geometrically organized into four domains. The first two domains along the chain resemble each other structurally and bind FAD and NADPH, respectively. The central and the interface domain follow at the COOH-terminal side.

Considering all residues making up one catalytic center one realizes that they come from five different domains: all four domains of one subunit and the interface domain of the other subunit. As compared to other known protein structures such a large number of domains participating in the catalytic center is unusual. Moreover, the catalytic center lies between subunits. For example both glutathione molecules are stretched between subunits as indicated in Fig. 2.

The catalytic center can be subdivided into two parts, one

**Table I**

<table>
<thead>
<tr>
<th>Soak</th>
<th>Ligand or reagent</th>
<th>Conc</th>
<th>Time</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NADPH</td>
<td>10 mM</td>
<td>36 min</td>
<td>7.0</td>
</tr>
<tr>
<td>B</td>
<td>NADPH⁺ and GSSG</td>
<td>10 mM and 10 mM</td>
<td>30 days</td>
<td>7.0</td>
</tr>
<tr>
<td>C</td>
<td>2-Mercaptoethanol</td>
<td>1% (w/v)</td>
<td>15 min</td>
<td>7.0</td>
</tr>
<tr>
<td>D</td>
<td>Dithioerythritol</td>
<td>2 mM</td>
<td>30 min</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Dithioerythritol</td>
<td>1 mM and 2 mM</td>
<td>150 min</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>and 2-iodoacetamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>NADPH⁺ Wash with storage buffer</td>
<td>100 mM</td>
<td>15 min</td>
<td>7.0</td>
</tr>
<tr>
<td>F</td>
<td>GSH and GSH</td>
<td>10 mM</td>
<td>3 days</td>
<td>8.5</td>
</tr>
</tbody>
</table>

**Fig. 1.** Stereo drawing of ligands bound to glutathione reductase together with important amino acid residues. The positions of FAD, NADPH, and GSSG correspond to those of Fig. 3, where they are named. The same applies for the depicted couples of amino acid side chains Cys-58:Cys-63, Lys-66:Glu-201, Arg-291:Asp-331 and His-467':Glu-472'. The side chain of Tyr-114 is given at its location without GSSG bound. On binding of this substrate the side chain of Tyr-114 moves by about 0.1 nm toward the viewer. All Cα-atoms of these amino acids are marked by dots. The bridge Cys-58:Cys-63 is given by a dotted line. The formation of the mixed disulfide Cys-58:Glutathione-I is marked by a dashed line.

**Fig. 2.** Gross structure of the dimeric enzyme glutathione reductase as viewed along the molecular 2-fold axis. The NH₂-terminal 18 residues are flexible. The remaining polypeptide chain is geometrically organized into four domains. The ligands FAD, NADPH, and GSSG are indicated. There exists a single intersubunit disulfide bridge across the molecular axis.
part binding NADPH and the other binding GSSG. This distinction reflects the two-step reaction catalyzed by the enzyme. As shown in Fig. 3 each part corresponds to a deep pocket in the protein, which is filled with solvent when the respective ligand is absent. These two pockets come from opposite sides of a subunit, and meet each other in the subunit center. If these pockets were not separated from each other by flavin and the redoxactive disulfide Cys-58:Cys-63, they would join and form a channel through the subunit. Binding of the substrates to the enzyme in such a geometry results in optimal protection of the reaction center against solvent molecules, which otherwise would interfere with the electron transfer process.

In Fig. 3 we have sketched all those parts of the enzyme-ligand complexes which are given as an exact drawing in Fig. 1. We find it advisable to describe the catalytic center using this sketch and refer the reader to Fig. 1 for exact geometry. The two dinucleotides FAD and NADPH bind in elongated conformations to the protein. The nicotinamide part of NADPH is in close contact with the flavin part of FAD, whereas the adenosine moieties of NADPH and FAD are far apart from each other; the distance between the N1 positions of the two adenines is 2.9 nm.

In the binding pocket of NADPH the side chain of Tyr-197 is indicated by a dashed line. Without NADPH being present this phenol ring covers the nicotinamide binding pocket and thus shields flavin against the solvent. On binding of NADPH (Soak A), but not on binding of NADP+(Soak B), Tyr-197 moves away so that nicotinamide can reach the flavin.

Flavin is surrounded by several pairs of polar, usually ionized residues, which most likely form salt bridges. The most conspicuous one is Lys-66:Glu-201 at the bottom of the nicotinamide binding pocket deeply buried in the protein. The imidazole of His-467' is fixed by a short and therefore strong salt bridge Lys-66:Glu-201. The distances are given in Fig. 4. Approximately in the same plane are the carbonyl oxygen of the amide side chain is above the pteridin moiety of flavin. The amide side chain is above the pteridin moiety of flavin. The exact geometry of the protein do not yield the positions of hydrogen atoms, only the charges and their ionization states of side chains are unknown. Only the charges of Cys-63 and His-467' have experimental support (see below).

The C4' atom of NADPH is close to N5 of flavin and to the salt bridge Lys-66:Glu-201. The distances are given in Fig. 4. Glutathione reductase belongs to the class of B-specific dehydrogenases (34), which means that the H2-atom is abstracted from C4' of NADPH. According to standard geometries this H2-atom lies at an intermediate level between the rings of flavin and nicotinamide. It is located at approximately equal distance to N5 of flavin and N4 of Lys-66. The H2-atom is placed between C4' and O41 of Glu-201. The observed distance between C4' and O41 is remarkably short.

In Fig. 5 we show the four states of the enzymatic reaction cycle, the geometries of which are known. Since x-ray analyses of proteins do not yield the positions of hydrogen atoms, all hydrogens given in Fig. 5 are guessed. In particular, the ionization states of side chains are unknown. Only the charges of Cys-63 and His-467' have experimental support (see below).

The oxidized enzyme, E, is represented as State 1 of Fig. 5. Its geometry is known at 0.2 nm resolution (24). The orientation of the imidazole ring of His-467' is established by the reasonable assumption that one of the nitrogens forms a hydrogen bond to the carboxyl group of Glu-472'. The distances between N4 of His-467' and the sulfurs of the closed
bridge Cys-58:Cys-63 are given in Table II.

State 2 of the reaction cycle given in Fig. 5 is the complex between NADP$^+$ and EH$_2$. In our experiments we have produced the complex between NADPH and EH$_2$ in Soak A because we used a large excess of reduced NADPH and because NADPH binds more tightly to EH$_2$ than NADP$^+$ (35). In both complexes, however, the NADP conformation and location are essentially the same, because the difference electron density maps of Soak A and of Soak D, where we produced the complex between NADP$^+$ and the blocked reduced enzyme EHR (carboxymethylated at Cys-58), correspond closely with each other at 0.3 nm resolution.

The 2-electron reduced form of the enzyme, EH$_2$, is represented as State 3 of Fig. 5. When compared to State I, the sulfur of Cys-58 has moved by about 0.1 nm. Furthermore, some positive difference electron density between the proximal sulfur of Cys-63 and flavin appears, indicating a slight movement of the order of 0.01 nm of this sulfur toward flavin on the formation of the charge transfer complex (32, 33). The distances between these two sulfurs and N$_i$ of His-467$^*$ are given in Table II.

In State 4 of Fig. 5 we merged three steps of the reaction, (i) the binding of GSSG to the reduced enzyme EH$_2$, (ii) the formation of the mixed disulfide between glutathione I and Cys-58 with subsequent release of glutathione II, and (iii) the release of glutathione I. This drawing is based on Soak B, which revealed how GSSG is bound to the oxidized enzyme E, and on Soaks E and F, which established that glutathione I forms the mixed disulfide and glutathione II leaves the enzyme first. In the mixed disulfide the sulfurs of glutathione I and Cys-58 are at essentially the same positions as in Soaks B and C, respectively. Although Soaks E and F show a certain although low occupancy of glutathione II, they cannot be located by x-ray diffraction. For distances see Table II.

![Image of Glutathione Reductase](https://example.com/glutathione_reductase_image.png)

**Fig. 4.** Environment of the stacked rings of flavin and nicotinamide. Flavin is assumed to lie in the paper plane with its face pointing to the viewer, all other atoms are projected vertically onto this plane. All distances are given in nanometers. Presumed hydrogen bonds are indicated by *rows of slashes*. Distance measurements are marked by *dashed lines*. Also in the paper plane is the carbonyl oxygen of His-467$^*$ and the center C$_n$ of the guanidinium group of Arg-291. The inserted $\alpha$-helix of residues 338–354 starts close to O$_2$ in the paper plane and extends to the upper rear side. The sulfur S$_i$ is located 0.34 nm below the paper plane and belongs to Cys-63. The nicotinamide ring lies 0.34 nm above the paper plane. Its carboxamide group is twisted as marked. The distance between O$_{\alpha}$ of Glu-201 and N$_i$ of Lys-66 is 0.24 nm. The distance between O$_{\alpha}$ and C$_{\alpha}^4$ of nicotinamide is 0.32 nm. The alkyl parts of Lys-66 covers tightly the N5 atom of flavin, the distance between C$_n$ and N5 being 0.34 nm. The accuracy of the nicotinamide position is about 0.05 nm.

[S$_i$ cannot be recognized safely. On formation of the mixed disulfide the sulfur of glutathione II moves away. The distances between the relevant atoms are given in Table II. Assuming that S$_i$ moves toward N$_i$ of His-467$^*$ where it can find space, and that it stops at van der Waals distance of 0.38 nm from its former sulfur partner S$_i$, the resulting distance to N$_i$ amounts to 0.38 nm. Thus, glutathione II may well pick up a proton from His-467$^*$. Moreover, there is no other conspicuous proton donor nearby.](https://example.com/glutathione_reductase_table.png)

**DISCUSSION**

Glutathione reductase is catalytically active in the crystals used for x-ray analysis; on addition of NADPH yellow crystals.
(State 1 of Fig. 5) turn red (State 3 of Fig. 5), and these red crystals turn yellow again when NADPH is removed and GSSG added. Therefore, the structurally analyzed intermediates are most likely catalytically competent.

The catalytic cycle starts with the binding of NADPH to the oxidized enzyme (State 1 of Fig. 5). As the first intermediate we define State 2 of Fig. 5, where the reduction equivalents have opened the redox-active disulfide bridge. In Soaks A and D (Table I) we produced the complexes EH2-NADPH and EHR-NADP+, which resemble each other closely. Therefore, it is most likely that within the limits of error their geometry corresponds to State 2 of Fig. 5.

The geometric arrangement of nicotinamide, flavin, and their environment are given in Fig. 4. Here, one has to keep in mind that x-ray analyses of proteins do not show hydrogen atoms. As a consequence all hydrogens given in, or discussed in conjunction with Figs. 4 and 5, are either postulated or derived from standard geometries. Fig. 4 demonstrates that the transferred H2-atoms of nicotinamide (34) is near to N5 of flavin and N6 of Lys-66. Since N5 is tightly covered by the alky l part of Lys-66, it is rather unlikely that H2 attaches to N5 and enters the flavin plane. Also, we cannot find any other reasonable pathway for H2 from the re- to the si-face of flavin. Thus, we conclude that H2 does not proceed to the redoxactive disulfide, but eventually remains as a proton in the NADPH-binding pocket (Figs. 3 and 5).

The nature and the sequence of the reduction equivalents transferred from NADPH to flavin and from flavin to the disulfide cannot be directly determined in an x-ray structure analysis. The observed geometry does not rule out previous suggestions (36-40): only the covalent bond between C2' and C4a proposed by Hemmerich (37) seems to be very unlikely. The atomic arrangement indicates that H2 ends up at N6 of Lys-66 after this has pushed one hydrogen over to Glu-201. The short distance between C4' and Oa of Glu-201 may help to drive electrons into flavin.

When immersed in the flavin ring system, the electrons have to be stabilized. A major contribution to this stabilization could come from the presumably positively charged residues around flavin (Fig. 3). Moreover, the NH2 terminus of a-helix 338-354 is near to N1 and O2a (Fig. 4). This helix stabilizes a negative charge, which occurs in this region in anionic flavin states (41).

For picking up the electrons from flavin the proximal sulfur of Cys-63 may form a short-lived covalent bond to C4a, as has been suggested from spectroscopic data (42). An intermediate bond between sulfur and C4a is likely to occur in the closely related enzyme lipoamide dehydrogenase (31). Moreover, such a bond has been observed in model studies (43, 44). After breaking this bond, Cys-63 forms a thiolate anion. This anion is at the NH2 terminus of a-helix 63-80 and therefore stabilized by the helix dipole.

In stopped flow experiments the formation of State 2 (Fig. 5) has been resolved into three steps (42). In the light of the then known general geometry at the active center (27), these steps have been interpreted as (i) a charge transfer complex between NADPH (donor) and oxidized flavin (acceptor), (ii) a charge transfer complex between NADP+ (acceptor) and reduced flavin (donor), and (iii) a covalent attachment of the proximal sulfur to C4a (42). The assumed charge transfer complexes, however, do not imply that electrons are passed on via such an interaction.

In the next step of the cycle the enzyme proceeds to State 3 (Fig. 5). The stable intermediate EH2 has been characterized in detail (1, 30). EH2 contains an oxidized flavin and a reduced disulfide. In this step NADP+ leaves its pocket. Presumably, this is facilitated by the surplus positive charge at Lys-66 repelling the positively charged nicotinamide. Furthermore, the additional proton in the salt bridge is released to the solvent via a column of water molecules, and the salt bridge is reinstalled.

From has been shown chemically (1, 30) the enzyme takes up 2 protons from the solvent when proceeding from State 1 to State 3. This happens most likely in the GSSG binding pocket. States 2 is drawn with these protons already present, although nothing is known about the time sequence of NADP+ detachment, salt bridge discharge, and proton uptake. For geometric reasons (Table II) the protons should be picked up one after the other using N6 of His-467 as a relay station. In EH2 a hydrogen bond between this N6 and S38 is very likely, whereas the distance between N6 and S58 is too large for such an interaction. Fig. 5 agrees well with chemical data, showing that a base with a pK of 6.5 has been protonated in EH2, which indicates that a histidine is involved (45). The charged histidine may also stabilize the thiolate anion.

The reactions from State 1 to State 3 (as well as the complete cycle) lead to a proton release in the NADPH pocket at one side of the enzyme (Fig. 3) and a proton uptake in the GSSG pocket at the other side. As a consequence, the enzyme would perform the function of a proton pump if it were suitably packed into a membrane (46, 47).

The stable intermediate EH2 allows the subdivision of the reaction cycle into the two parts given above as separate equations. The structure analysis shows that these two parts are also geometrically distinct from each other (Figs. 2, 3, and 5). Between State 1 and State 3 of Fig. 5 the most essential reactions happen at the NADPH site, i.e., at the re-face of flavin, whereas the following bridge exchange takes place at the si-face. The degree of separation is also reflected in the observation that transhydrogenase and electron transferase activity, which are by-reactions occurring in the NADPH pocket, are not much affected by enzyme modifications in the GSSG pocket (47).

When the substrate GSSG is bound, the enzyme proceeds to State 4. This figure combines three reaction steps: (i) the binding of GSSG to EH2, (ii) the formation of the mixed disulfide with subsequent release of glutathione II, and (iii) the release of glutathione I. Our x-ray analysis showed how GSSG is bound to the oxidized enzyme (Soak B). Since the only appreciable difference between EH2 (State 3) and E (State 1) is the opened disulfide bridge, we can safely use Soaks B and C and derive the structure of the complex EH2-GSSG from E-GSSG and EH2.

On binding of GSSG, the phenol ring of Tyr-114 moves by about 0.1 nm and accommodates itself snugly between the glycine moieties of glutathione I and II. The tyrosine hydroxyl group is at van der Waals contact to both sulfurs of GSSG (Fig. 5). However, because of its high pK value we hesitate to suggest this hydroxyl as a proton donor for any one of the leaving glutathiones. We do not expect that this pK is lowered in the rather nonpolar environment of the phenol.

From Soaks E and F we know that glutathione I forms a mixed disulfide with the protein, E-SG, and that glutathione II leaves the enzyme first. This order could have been guessed from the structure of E-GSSG bound with Soak H, because in this complex glutathione I is much more tightly bound than glutathione II. The crystals of Soaks E and F have the red color characteristic for EH2, indicating that the charge transfer complex between thiolate anion and flavin is still intact. As shown in Table II, the opening of the Cys-58:Cys-63 bridge makes S38 move toward S1 (of glutathione I) the distance decreasing from 0.50 to 0.36 nm. At its new position S38 can start a nucleophilic attack on S1, while the proton of S58 leaves its position between S38 and S58 and moves towards N6 of His-

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Concomitantly, the proton of N to SII, which on breaking of the S-I-SI bond can come as near as 0.38 nm to N,. Glutathione I is then released. Since SI of this disulfide is close to N, (Table II), SI is likely to receive the (second) proton from His-467'. Glutathione I is detached, and His-467' returns to its unprotonated state. Presumably, the imidazole ring of His-467' does not move in the catalytic process because it is firmly held by Ghu-472'.

The described sequence of events in State 4 of Fig. 5 has already been suggested on the basis of chemical data (1, 29). The structure analyses contribute here by providing rather exact geometric relations and by identifying the reaction partners in GSSG and in the enzyme.

In conclusion we report here the x-ray structures of E, complex EH2-NADP* (as inferred from EHR-NADP* and EHA-NADPH), EH2 complex EH2-GSSG (as inferred from E-GSSG and EH2), and E-SG, which constitute the steps of the reaction cycle given in Fig. 5. These analyses did not reveal any hydrogens. Therefore, the ionic states of possible charged residues remain unknown. As an exception the thiolate of Cys-58 (32, 33) and the charge of His-467' in EH2 (45) have gained some experimental support. In the future we shall reveal any hydrogens. Therefore, the ionic states of possible charged residues remain unknown. As an exception the thiolate of Cys-58 (32, 33) and the charge of His-467' in EH2 (45)

Acknowledgment—We thank Dr. R. Heiner Schirmer for providing us with a pure glutathione reductase preparation ready for crystallization as well as for very informative discussions, and Dr. Alwyn Jones for generous help on the interactive display system at Uppsal, Sweden.

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Catalytic Mechanism of Glutathione Reductase 1757
The catalytic mechanism of glutathione reductase as derived from x-ray
diffraction analyses of reaction intermediates.

E F Pai and G E Schulz


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