We have determined the crystal structure of a complex between the noncompetitive inhibitor (K$_{i}$ = 27 µM, K$_{ii}$ = 48 µM with respect to oxidized glutathione (GSSG) and K$_{i}$ = 144 µM, K$_{ii}$ = 176 µM with respect to NADPH) 6-hydroxy-3-oxo-3H-xanthene-9-propionic acid (XAN) and human glutathione reductase (hGR). The structure, refined to an R-factor of 0.158 at 2.0 Å resolution, reveals XAN bound in the large cavity present at the hGR dimer interface where it does not overlap the glutathione binding site. The inhibitor binding causes extensive local structural changes that primarily involve amino acid residues from a 30-residue α-helix that lines the cavity and contributes to the active site of hGR. Despite the lack of physical overlap of XAN with the GSSG binding site, no GSSG binding is seen in soaks carried out with high XAN and GSSG concentrations, suggesting that some subtle interaction between the sites exists. An earlier crystallographic analysis on the complex between hGR and 3,7-diamino-2,8-dimethyl-5-phenyl-phena-zinium chloride (safranin) showed that safranin bound at this same site. We have found that safranin also inhibits hGR in a noncompetitive fashion, but it binds about 16 times less tightly (K$_{i}$ = 453 µM, K$_{ii}$ = 586 µM with respect to GSSG) than XAN and does not preclude the binding of GSSG in the crystal. Although in structure-based drug design competitive inhibitors are usually targeted, XAN’s binding to a well-defined site that is unique to glutathione reductase suggests that noncompetitive inhibitors could also serve as lead compounds for structure-based drug design, in particular as components of chimeric inhibitors.

Intracellular parasites such as the P. falciparum require a highly efficient thiol metabolism to protect themselves from intracellular reactive oxygen species and their derivatives (3). This implies that they are highly susceptible to oxidative stress and that this sensitivity is a promising target for drug action (4, 5). The role of oxidative stress as an important mechanism for the destruction of parasites and tumor cells (6) is particularly well illustrated by many congenital and acquired factors that generate oxidative stress in human erythrocytes and offer partial protection against malaria (4). Central to defense against intracellular oxidative stress in humans is the glutathione redox cycle, which involves the enzymes human glutathione reductase (hGR) and glutathione peroxidase. The flavoenzyme hGR catalyzes the NADPH-dependent recycling of oxidized glutathione (GSSG) to maintain high levels of reduced glutathione as shown by the equation below (7).

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightleftharpoons 2\text{GSH} + \text{NADP}^+ \quad \text{(Eq. 1)}$$

Despite the importance of hGR, there is good evidence that hGR is not essential for normal erythrocyte function and that the reduced life-span of hGR-deficient red blood cells is tolerable (8). As reviewed by Schirmer et al. (2), hGR is thus a reasonable target for rational drug design. In this direction, various compounds have been reported to inhibit hGR (9–11).

In choosing hGR as a target for structure-based drug design, we have considered, in addition to the enzyme’s physiological importance, the plethora of structural information that is available; the three-dimensional structure of hGR has been solved and refined to 1.54 Å resolution, and its catalytic mechanism has been well established from crystal structures of the enzyme complexed with its natural substrates GSSG and NADPH, substrate analogues, and various other ligands (12–17). Furthermore, the gene for hGR has been cloned and overexpressed in Escherichia coli, which makes possible the study of structure-function relations through the generation of site-directed mutants (18).

In a recent study on the prediction of ligand binding to proteins by affinity fingerprinting methods (19), yeast glutathione reductase was used as one of the model protein drug targets. The study identified a number of tight binding inhibitors from a variety of chemical families, yet their modes of action were not investigated. Because yeast glutathione reductase and hGR are homologous (48% overall amino acid sequence identity), we have tested some of the yeast GR inhibitors as inhibitors of hGR and found that they exhibit diverse modes of inhibition. One of them, 6-hydroxy-3-oxo-3H-xanthene-9-propionic acid (XAN), is a noncompetitive inhibitor that binds in a cavity of hGR where the dye 3,7-diamino-2,8-

**References**

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2. The atomic coordinates and structure factors (codes 1XAN and R1XANSF) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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4. In a recent study on the prediction of ligand binding to proteins by affinity fingerprinting methods (19), yeast glutathione reductase was used as one of the model protein drug targets. The study identified a number of tight binding inhibitors from a variety of chemical families, yet their modes of action were not investigated. Because yeast glutathione reductase and hGR are homologous (48% overall amino acid sequence identity), we have tested some of the yeast GR inhibitors as inhibitors of hGR and found that they exhibit diverse modes of inhibition. One of them, 6-hydroxy-3-oxo-3H-xanthene-9-propionic acid (XAN), is a noncompetitive inhibitor that binds in a cavity of hGR where the dye 3,7-diamino-2,8-
hGR in Complex with Xanthene Inhibitor

dimethyl-5-phenyl-phenazinium chloride (safranin) had been previously seen to bind (13). As a result of this finding we have also assessed the inhibition of hGR by safranin, which also behaved as a noncompetitive inhibitor with respect to GSSG, yet its inhibition potency was 16 times weaker than XAN. Here, we report on the inhibition of hGR by these two compounds and on the crystal structure of hGR in complex with XAN at 2.0 Å resolution.

**EXPERIMENTAL PROCEDURES**

**General Materials—** All reagents used were of the highest purity available and were purchased from Aldrich, Fisher, Sigma, and Amicon.

**Enzyme Preparation—** An overexpression system for hGR (18, 20) was kindly provided by Dr. Dieter Werner and Dr. Heiner Schirmer (University of Heidelberg, Germany). A preparation protocol for the recombinant enzyme has been published (21), but for our preparations (10-liter cell cultures), we found that additional steps were essential to obtain highly pure hGR. Our purification scheme is briefly described here.

Plasmid-carrying E. coli SGS cells, which had been grown for ~14 h (65 nm), in 10 liters of two times concentrated trypotene-yeast extract medium containing 0.1 mg/ml ampicillin, were harvested by centrifugation. The cells were suspended in lysis buffer (0.1 M Hepes, pH 7.2, 1 mM EDTA, 50 mM β-mercaptoethanol), containing 0.1 mM phenylmethylsulfonyl fluoride and 0.05 mg/ml FAD and were lysed by French Press (12000 psi) and sonication. The crude extract was clarified by centrifugation (25,900 g) and dialyzed against 10 ml of buffer B. The dialysate was then dialyzed against buffer B (20 mM Tris, 1 mM EDTA, 5 mM KCl, and 5 mM β-mercaptoethanol, pH 8.0) and applied to a 50-ml column of 5′-ADP-Sepharose equilibrated with buffer A, the inhibitory buffer, which was eluted with 30 ml of 1 mM NADPH in buffer A. The enzyme was reoxidized and stabilized by the addition of 2 ml GSSG. The solution was then dialyzed against buffer B (20 mM Tris, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 8.5) and applied to a 30-ml column of DEAE-Sephadex equilibrated with buffer B. After washing with 150 ml of buffer B, the enzyme was eluted with a linear salt gradient (0–0.4 M KCl in buffer B). The active fractions were pooled and dialyzed against buffer B. The dialyzed protein solution was loaded onto a 10-ml column of Procion Red HE-3B and washed extensively with buffer B. Pure hGR was eluted with a linear salt gradient of 0–2 M KCl in buffer B. Fractions containing hGR were pooled, concentrated to 15 mg/ml, and dialyzed against storage buffer (3% ammonium sulfate, 0.1 M potassium phosphate, pH 7.0).

The final concentration of the enzyme was assessed using ε₄₅₀ = 11.3 mM⁻¹ cm⁻¹ (22). Enzyme activity was measured using two different substrates (23). The yields per liter of cell culture were 3–4 mg of electrophoretically homogeneous (~98% pure) hGR, with an A₄₅₀/A₅₉₅ ratio of 6.6. The Kₘ for GSSG of 65 µM, and the Kₘ for 12,500 min⁻¹ agree well with those reported by Nordhoff et al. (21) for recombinant hGR and by Worthington and Rosemeyer (24) for the native enzyme.

Inhibitors—Safranin was purchased from Aldrich, XAN was a gift from Terrapin Technologies Inc. (Menlo Park, CA). Chemical structures for both compounds are presented in Fig. 1.

**Steady State Kinetics and Inhibition Studies—** All enzyme kinetics experiments were carried out at room temperature (298 K) using Shimadzu® (UV1600U) and Otsu® UV-visible spectrophotometers. Enzyme activity was spectrophotometrically monitored from the rate of conversion of NADPH to NADP⁺ (ε₅₄₀ = 6.2 mM⁻¹ cm⁻¹). For each assay the absorbance of NADPH in the reaction mixture was measured over a period of 1 min.

Assays were carried out in 1 ml of the following solution: 0.2 M KCl, 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA, 0.1 M NADPH, 30–1000 µM GSSG, 0.6 M hGR, and 35 µM bovine serum albumin. The added bovine serum albumin stabilized hGR, which otherwise rapidly lost activity at the low concentration used in the assay (22). For the inhibition studies, inhibitors were added to the above mixture at the desired concentrations, whereas all other parameters stayed the same. In the case of XAN, where the inhibitor was dissolved in 0.1 M Tris at pH 8, control assays were performed in the presence of Tris. For each inhibitor we used three different concentrations. The inhibitor concentrations were kept constant, and activity measurements were made at 30–1000 µM GSSG. For XAN we have also carried out activity measurements by keeping a saturating 1 mM concentration of GSSG and taking measurements at 5–100 µM NADPH. The reaction was initiated by the addition of NADPH once all other reagents were present in the assay mixture.

The inhibition constants, Kᵢ for noncompetitive inhibitors were estimated by fitting the data to the following equation using the FORTRAN programs of Cleland (25).

![Equation](https://latex.codecogs.com/svg.latex?\begin{align*}
  v = \frac{V_{max}[S]}{K_m (1 + \frac{[I]}{K_i}) + [S] (1 + \frac{[I]}{K_i})}
\end{align*})

where [S] is the concentration of the variable substrate, [I] is the concentration of the inhibitor, Kᵢ is the inhibition constant, and for pure competitive inhibition Kᵢ is infinity.

**Crystallization—** Crystals of recombinant hGR suitable for crystallographic analysis were reproducibly grown at room temperature using 10-µl hanging drops containing recombinant hGR at 20 mg/ml, 3% ammonium sulfate, 0.1 M potassium phosphate, pH 7.0, and 0.1% β-oc-tyl-glucoside. The drops were equilibrated against a 700-µl reservoir containing 21–23% ammonium sulfate and 0.1 M potassium phosphate, pH 8. Diffraction quality monodonic crystals (0.5 × 0.5 × 0.4 mm) of space group C2 and unit cell parameters, a = 119.7 Å, b = 63.35 Å, c = 84.65 Å, α = γ = 90° and β = 58.61°, were grown in 2 days. Although our crystallization conditions are rather different from those used to crystallize natural hGR (26), the crystals are equivalent to those used for the high resolution studies of natural hGR (22), but in order to follow crystallographic conventions we have indexed them in space group C2 rather than B2.

**Inhibitor Soaking Experiments—** Crystals of hGR were stored and handled in artificial mother liquor containing 25% ammonium sulfate in 0.1 M potassium phosphate, pH 8.0. To increase the solubility of XAN in the conditions of the artificial mother liquor, the inhibitor was first dissolved in 0.1 M Tris at pH 8.0 then used for the soaking experiments. Because aqueous solutions of XAN and safranin are orange-red and dark red, respectively, we observed a marked change in the color of the crystals at the end of soaking experiments involving these two compounds. Crystals soaked for conditions for our crystallographic studies are presented in Table I.

**X-ray Data Collection—** All x-ray data were collected on San Diego MacScience area detectors with a Rigaku RU-200 rotating anode (CuKα) and a graphite monochromator. The generator operated at 50 kV/150 mA⁻¹ with a 0.5 × 5-mm focus. Data collection and reduction were carried out as recommended (27, 28). Data collection statistics are shown in Table I.

**Model Building and Structural Refinement—** The structure for recombinant hGR was refined at 2.0 Å resolution by the conventional positional refinement routines in XPLOR (29). The refined structure of the naturally occurring enzyme (Ref. 12; Protein Data Bank entry 1hgr) was used as the starting model after coordinates were altered to comply with our designation of the space group C2.

The model for XAN was built using the ChemNote utility of QUANTA (Molecular Simulations, Inc.) and minimized using CHARMM (Molecular Simulations, Inc.). Binding of XAN to the enzyme was determined in difference electron density maps, and coefficients (F_calcd - F_observed) and calculated phases from the refinement of the native structure (F_observed). Initially, data set XAN-1 at 2.3 Å resolution (Table I) was used. Difference electron density maps showed incomplete density for the tricyclic xanthene moiety of the molecule, but after one round of positional refinement using the native hGR structure after removal of water molecules in the XAN binding site, the density improved. XAN was then fitted, and manual changes to the protein model were made using the program CHAIN (30). Further cycles of conventional positional refinement against data between 10 and 2.3 Å resolution, and manual fitting yielded a structure with an R-factor of 0.143. At this point data were collected from crystals equilibrated in artificial mother liquor containing XAN and GSSG (data set XAN-2, Table I). Because binding of GSSG was not observed, data sets XAN-1 and XAN-2 were merged to generate a more complete 2.0 Å resolution data set (Table I). Several further rounds of manual retitling and positional refinement resulted in our final model of the complex with an R-factor of 0.158 for all data between 10 and 2.0 Å resolution.
RESULTS AND DISCUSSION

Kinetic studies showed that XAN inhibition of hGR is non-competitive with respect to GSSG, with $K_{i_s} = 27 \mu M$ and $K_{i_i} = 48 \mu M$ (see Fig. 2). Additional experiments at saturating concentrations of GSSG and varying concentrations of NADPH revealed that XAN is also noncompetitive to NADPH with $K_{i_s} = 144 \mu M$ and $K_{i_i} = 176 \mu M$ (data not shown). This mode of inhibition suggests that XAN binds reversibly both to the free enzyme and the enzyme-substrate complex at a site distinct from that of the natural substrate(s). Furthermore, assays done in the absence of GSSG showed that XAN itself does not act as an electron acceptor. The observed noncompetitive inhibition of XAN shows that the diverse compounds identified by the affinity fingerprinting approach to drug design (19) may bind to various sites on the enzyme. Given this extra degree of freedom, the predictive success of this study makes it all the more noteworthy.

The catalytic mechanism of hGR is a bi-bi ping-pong mechanism with NADPH reacting first to produce the reduced enzyme (EH$_2$), which in turn binds and reduces GSSG (14, 15):

reductive half reaction:

$$E + \text{NADPH} \rightarrow E \cdot \text{NADPH} \rightarrow \text{EH}_2 \cdot \text{NADP}^+ \rightarrow \text{EH}_2$$  (Eq. 3)

oxidative half reaction:

$$\text{EH}_2 + \text{GSSG} \rightarrow \text{EH}_2 \cdot \text{GSSG} \rightarrow \text{ESSG} \cdot \text{GSH} \rightarrow E \cdot \text{GSH} \cdot \text{GSH} \rightarrow E$$  (Eq. 4)

Because there are more than just the E and ES forms of the enzyme present, the $K_i$ values need not be exactly equated with single dissociation constants. Nevertheless, two factors suggest that there is an element of XAN inhibition that is competitive with GSSG. First is the 5-fold poorer inhibition by XAN in the presence of saturating GSSG ($K_{i_s} = 144 \mu M$) compared with the presence of saturating NADPH ($K_{i_i} = 27 \mu M$), and second is the 1.8-fold difference in $K_{i_s}$ versus $K_{i_i}$ (48 $\mu M$ versus 27 $\mu M$) with respect to GSSG. Both observations suggest that GSSG-bound forms of the enzyme bind to XAN more weakly, which directly implies that XAN bound forms of the enzyme bind GSSG more weakly.

After our crystallographic studies (see below) showed that XAN bound in a site known to bind safranin (13) (Fig. 1), we

![Chemical structures of XAN and safranin.](http://www.jbc.org/)

**TABLE 1**

| Crystal   | Conditions               | Resolution limit | Unique reflections (multiplicity) | $R_{sym}$ | % complete |
|-----------|--------------------------|------------------|----------------------------------|-----------|------------|
| Native    |                          | 2.0              | 34,095 (2.1)                     | 0.061     | 93         |
| XAN-1     | 1.3 mM XAN, 0.4 mM Tris, pH 8.0, 12-h soak | 2.3              | 23,398 (4.1)                     | 0.068     | 96         |
| XAN-2     | 2.0 mM XAN, 10 mM GSSG, 0.4 mM Tris, pH 8.0, 3-day soak | 2.0              | 31,995 (2.6)                     | 0.046     | 87         |
| XAN-3     | Merged XAN-1 and XAN-2 data sets | 2.0              | 33,736 (5.4)                     | 0.071     | 92         |
| SAF-1     | Safranin (saturated, ~10 mM), 10 mM GSSG, 7-day soak | 2.0              | 35,937 (3.1)                     | 0.047     | 94         |

$$R_{sym} = \frac{\sum_{h} \sum_{i} |I(h)| - \langle |I(h)| \rangle}{\sum_{i} |I(h)|}$$
tested the inhibitory effects of safranin. Safranin also behaved as a noncompetitive inhibitor with respect to GSSG (Fig. 2) but with markedly less potency than XAN, having $K_{i} = 453 \, \mu M$ and $K_{ii} = 586 \, \mu M$ with respect to GSSG.

To characterize the mode of binding and the inhibition mechanism of XAN and safranin, we have analyzed crystal structures of hGR soaked with XAN alone, GSSG alone, a combination of XAN and GSSG, and a combination of safranin and GSSG (Table I).

XAN binds at the crystallographic 2-fold axis in the large cavity at the dimer interface of hGR with a stoichiometry of one XAN molecule:hGR dimer. The cavity has been previously identified as one that binds safranin and menadione and to a lesser degree lipoate, $\alpha$-$\gamma$-GSSG, and S-(2, 4-dinitrophenyl)-glutathione, but whether it has a biological function is still unknown. The cavity connects to both GSSG binding sites through two channels that also serve as mediators between the bulk solvent and the cavity (13). Structural refinement using the conventional positional refinement routines in X-PLOR yielded our final model for the hGR-XAN complex at 2.0 Å resolution, which has an R-factor = 0.158. Fig. 3A shows the electron density maps in the region of the XAN binding site including the refined model for the inhibitor molecule. The electron density for the carboxylate group of the inhibitor is lower because each position of the carboxylate is only present at half occupancy, whereas the remaining atoms of the inhibitor overlap with their symmetry mates, effectively bringing them to full occupancy. At the same time, however, it is worth noting the remarkable ordering of the residues involved in the binding of XAN, which is manifested in the low B-factors (main chain and side chain atoms). In the unliganded structure of hGR, this region exhibits high disorder with B-factors in the 40–60 Å$^2$ range. B, $\pi$ electron-$\pi$ electron interactions between XAN and Phe$^{78}$. Shown is an excerpt from the final electron density maps in the region of the XAN binding site after refinement of the hGR-XAN complex at 2.0 Å resolution shown in A. We have modelled XAN with its xanthene ring being planar based on the fact that the structures of fluorescein and erythrosine B ethanolate (which also have a xanthene moiety) also exhibit planarity of their tricycle (30, 31). The irregular electron density for the tricycle is consistent with the relatively high B-factors of the atoms in the xanthene moiety (35–40 Å$^2$) and with some slight puckering of the xanthene.

**Fig. 3.** A, quality of the electron density maps. The final $2F_o - F_c$ electron density after refinement of the hGR-XAN complex at 2.0 Å resolution is shown. The map is contoured at 1.0 $\sigma$ level. Shown is the region of the XAN binding site including the refined model for the inhibitor molecule. The electron density for the carboxylate group of the inhibitor is lower because each position of the carboxylate is only present at half occupancy, whereas the remaining atoms of the inhibitor overlap with their symmetry mates, effectively bringing them to full occupancy. At the same time, however, it is worth noting the remarkable ordering of the residues involved in the binding of XAN, which is manifested in the low B-factors (main chain and side chain atoms). In the unliganded structure of hGR, this region exhibits high disorder with B-factors in the 40–60 Å$^2$ range. B, $\pi$ electron-$\pi$ electron interactions between XAN and Phe$^{78}$. Shown is an excerpt from the final electron density maps in the region of the XAN binding site after refinement of the hGR-XAN complex at 2.0 Å resolution shown in A. We have modelled XAN with its xanthene ring being planar based on the fact that the structures of fluorescein and erythrosine B ethanolate (which also have a xanthene moiety) also exhibit planarity of their tricycle (30, 31). The irregular electron density for the tricycle is consistent with the relatively high B-factors of the atoms in the xanthene moiety (35–40 Å$^2$) and with some slight puckering of the xanthene.
and Met\textsuperscript{79}, for which the possibility that they exist in alternate conformations has been suggested (12). Upon binding of XAN, however, these residues, along with His\textsuperscript{75} and His\textsuperscript{82} reorient substantially (Fig. 4A) and become quite rigid with B-factors dropping to near 15 Å\textsuperscript{2}.

An additional structural change involves Cys\textsuperscript{90} and Cys\textsuperscript{90'}, which form an intersubunit disulfide bridge in the native hGR structure. In the hGR-XAN complex the electron density for the disulfide bridge decreases and elongates (Fig. 3A), indicating increased mobility of this group (along with residues 88–94) and possibly an opening of the disulfide in a fraction of the molecules. In the liganded structure, residues 88–94 have the highest B-factors in the structure and make up the only region whose B-factors are higher in the XAN-bound structure than in the XAN-free state.

The observed conformational changes result in a hydrogen bond network involving the inhibitor and neighboring amino acid residues (Fig. 4B). In the native hGR structure, N\textsuperscript{δ1} of His\textsuperscript{75} makes a short (2.6 Å) hydrogen bond with N\textsuperscript{δ1} of His\textsuperscript{82}. In the XAN-bound structure the two residues move apart (Fig. 4A) so that each one can form a hydrogen bond with the oxygen on the carboxylate group of the inhibitor. His\textsuperscript{82} and His\textsuperscript{82'} make additional hydrogen bonds with the reoriented Cys\textsuperscript{90} and Cys\textsuperscript{90'}, which further support a partially open conformation for the disulfide bridge between Cys\textsuperscript{90} and Cys\textsuperscript{90'}. His\textsuperscript{75} is now able to form a new hydrogen bond (2.8 Å) with Tyr\textsuperscript{407}. A hydrogen bond between Tyr\textsuperscript{407} and Asp\textsuperscript{81}, which is present in unliganded hGR, is maintained in the hGR-XAN complex despite the fact that both these residues have shifted slightly. The tricyclic moiety participates in the hydrogen bonding network through interactions of its oxygens at positions 15 and 16 with Water\textsubscript{358}, Asn\textsuperscript{71} and their symmetry-related partners. Moreover, the central oxygen at position 10 of the xanthene makes a rather long hydrogen bond to Water\textsubscript{435}. Non-
polar interactions between hGR and XAN are made primarily by Phe$^{78}$ and Phe$^{79}$, which align almost parallel to and about 4 Å away from the plane of the inhibitor’s tricycle forming a “sandwich” type of interaction (Fig. 3B).

Surprisingly, the binding site is in a region that is not well conserved between human and yeast GR. Among the residues involved in the binding site (Fig. 4B), none is conserved. Although we still think it is likely that XAN binds at an equivalent site in yeast GR, this need not be so, and even if it does many details of the binding have to be different. In this light we must view the similarity of the inhibition constants of XAN for yeast GR and hGR as fortuitous.

To characterize the effects of XAN binding on GSSG binding, hGR crystals were soaked in artificial mother liquor containing both compounds (XAN-2, Table I). In this soak only XAN was seen to bind, and a control soak with all components of XAN-2 but inhibitor showed that GSSG bound at high occupancy, thus eliminating the possibility that the buffer of the soak hampers binding of GSSG. Interestingly, when we attempted to obtain a double complex containing safranin and GSSG (SAF-1, Table I), we observed binding of both compounds. The lack of GSSG binding in the XAN-2 soak surprised us because the kinetics had only suggested that XAN was slightly competitive with respect to GSSG (see above). One possible explanation is that under the conditions of the crystalline enzyme (e.g., high ionic strength), the XAN-hGR interaction changes so that the binding of XAN directly interferes with GSSG binding. However, the results for safranin make this hypothesis less attractive.

Another possibility is that the binding of XAN does indeed decrease the binding affinity of GSSG but that this is not visible in the kinetics because of the complexity of the two-substrate hGR reaction (33). We note in this regard that for similar reasons the aldose reductase inhibitor zopolrestat behaves kinetically as a noncompetitive inhibitor but is crystallographically seen to block the aldose binding site (34).

Despite our relatively detailed crystallographic results pertaining to the interactions XAN makes with the enzyme, the inhibition mechanism remains unclear. Based on the observed noncompetitive inhibition, we expected to see structural changes that might affect placement of active site residues or alter the mode of GSSG binding without hindering the affinity of GSSG to the active site. In this way catalysis could be impaired in a manner that could not be overcome by high concentrations of GSSG. In the hGR-XAN complex, XAN is located about 20 Å from the redox-active disulfide of hGR and is about 10 Å from the nearest atom of GSSG in its expected binding mode. This indicates that the observed inhibition is not due to a direct interaction between the inhibitor and the active site of the enzyme (Fig. 5). We note that most residues changing conformation or directly interacting with XAN (His$^{75}$, Phe$^{78}$, Met$^{79}$, Asp$^{81}$, and His$^{82}$) reside on the long α-helix that contains the active site disulfide. Despite this fact, no significant movements of atoms in or near the active site disulfide can be observed. The only structural effects that can be seen near the active site are small scale main chain shifts close to the GSSG binding site and in regions that directly interact with GSSG. The most prominent of these is a 0.3 Å shift of Met$^{406}$ whose main chain nitrogen donates a hydrogen bond to the γ-Glu-II-OT of GSSG in the hGR-GSSG complex (14). Although it would be surprising, it is conceivable that these small conformational changes coupled with the observed decrease in the mobility of the crystalline enzyme could affect binding of GSSG and catalysis. Studies that have been carried out using crystalline ribonuclease A as a model have suggested that enzyme flexibility is required for catalytic function and that a decrease in enzyme flexibility can hamper the activity of the enzyme (35). Some evidence against this hypothesis is provided by safranin, which also inhibits hGR but does not produce extensive conformational changes either in the hGR-safranin complex (13) or in our hGR-safranin-GSSG structure. Phe$^{78}$ and Phe$^{79}$ are the only residues that are markedly affected by the binding of safranin. An alternative explanation for our results is that the crystallographically seen binding site is a passive one not responsible for inhibition and that the true inhibitory site has not been observed in the crystals. Although this is possible, we think it is unlikely because the XAN binding site is adjacent to the GSSG binding site and is known to be highly occupied for compounds that show this type of inhibition.

Although we cannot presently detail the mechanism by which XAN and safranin inhibit hGR, we know of no other noncovalent, noncompetitive inhibitors that do not occupy the active site and for which the structural mechanism of inhibition is known. In this light, further investigation of the mechanism of inhibition of these inhibitors should be valuable. We expect that calorimetric analyses, and fast reaction kinetics will shed more light on the inhibition mechanism of XAN and safranin.

The residues lining the XAN binding pocket in hGR are not well conserved among homologs of hGR, so although compounds binding at this site are not competitive inhibitors, they are reasonable for structure-based drug design. In this regard, earlier results by Becker et al. (9) and more recent work by Kirsch et al. (36) have focussed on flavin derivatives that are hGR inhibitors and are expected to bind at the same dimer interface cavity that XAN and safranin do. In the case of XAN we see possibilities to rationally improve the potency of the compound. The enzyme-inhibitor interactions are tight in many regions, but there is room to make more extensive interactions through the placement of substituents of appropriate length at positions 3, 4, 5, and 6 of the tricyclic moiety of XAN. Furthermore, a replacement of XAN’s carboxylate group with
amide or hydroxyl groups might provide more optimal interactions with the surrounding environment, and at the same time it would also test the importance of the negatively charged tail of XAN for inhibition and binding.

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