Biochemistry

 Volume 32, Number 48 December 7, 1993

Accelerated Publications

Snapshots along the Reaction Coordinate of an S_NAr Reaction Catalyzed by Glutathione Transferase[†,‡]

Xinhua Ji,[§,∥] Richard N. Armstrong,[*,§] and Gary L. Gilliland[*,∥,⊥]

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, and Center for Advanced Research in Biotechnology of the Maryland Biotechnology Institute, University of Maryland, Shady Grove, and of the National Institute of Standards and Technology, 9600 Gudelsky Drive, Rockville, Maryland 20850

Received August 19, 1993; Revised Manuscript Received October 1, 1993[⊗]

ABSTRACT: The three-dimensional structures of a class *mu* glutathione transferase in complex with a transition-state analogue, 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate, and a product, 1-(*S*-glutathionyl)-2,4-dinitrobenzene, of a nucleophilic aromatic substitution (S_NAr) reaction have been determined at 1.9- and 2.0-Å resolution, respectively. The two structures represent snapshots along the reaction coordinate for the enzyme-catalyzed reaction of glutathione with 1-chloro-2,4-dinitrobenzene and reveal specific interactions between the enzyme, intermediate, and product that are important in catalysis. The geometries of the intermediate and product are used to postulate reaction coordinate motion during catalysis.

The cytosolic glutathione *S*-transferases catalyze the addition of the tripeptide glutathione (GSH)[1] to compounds bearing a wide variety of electrophilic functional groups (Armstrong, 1991, 1993; Rushmore & Pickett, 1993). This generic reaction is crucial in initiating the metabolism, transport, and detoxification of potentially toxic alkylating agents in vertebrates, plants, insects, and aerobic microorganisms. Most GSH transferases exhibit very broad substrate specificity and catalyze a variety of reaction types, including epoxide ring openings, Michael additions, and nucleophilic aromatic substitution (S_NAr) reactions. In fact, a reaction of the latter type, the addition of GSH and 1-chloro-2,4-dinitrobenzene (CDNB), shown in Scheme I, is the basis for the most widely used spectrophotometric assay of the enzyme (Habig et al., 1974).

Reactions of the S_NAr type are generally considered to proceed in solution via formation of a Meisenheimer-complex or σ-complex intermediate (Miller, 1968; Bernasconi, 1980) as illustrated in Scheme I. It appears that with many nucleophiles and leaving groups the rate-limiting step of the reaction is the formation of the intermediate. Kinetic evidence suggests that the enzyme-catalyzed addition of GSH to electron-deficient arenes proceeds with rate-limiting formation of the σ-complex intermediate (Chen et al., 1988). Thus, it might be anticipated that an enzyme capable of catalyzing such a reaction would also stabilize the σ-complex intermediate, at least to the extent that the intermediate resembles the transition state for its formation. However, the instability of 1-chloro-1-(*S*-glutathionyl)-2,4-dinitrocyclohexadienate ($GSCDCD^-$, Scheme I) has thus far precluded its detection. In contrast, the reversible reaction of GSH with 1,3,5-trinitrobenzene to form the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate anion ($GSTCD^-$), a potent inhibitor of the enzyme, provides a relatively stable model for this intermediate (Clark & Sinclair, 1988; Graminski et al., 1989), as shown

[†] Supported by a grant (GM30910) from the National Institutes of Health.

[‡] The final coordinates for the transition state analogue and product structures have been deposited in the Brookhaven Protein Data Bank under the file names 4GST and 5GST, respectively.

[*] Address correspondence to these authors.

[§] University of Maryland, College Park.

[∥] Center for Advanced Research in Biotechnology.

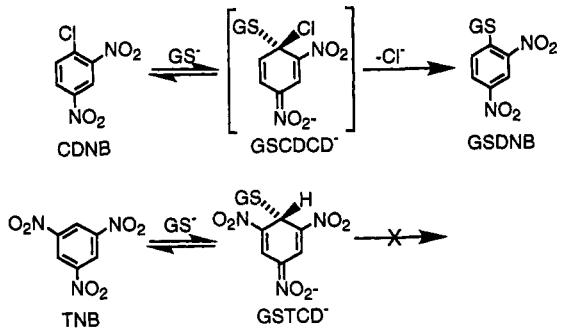
[⊥] National Institute of Standards and Technology.

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1993.

[1] Abbreviations: GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; TNB, 1,3,5-trinitrobenzene; $GSTCD^-$, 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate anion; $GSCDCD^-$, 1-chloro-1-(*S*-glutathionyl)-2,4-dinitrocyclohexadienate anion; GSDNB, 1-(*S*-glutathionyl)-2,4-dinitrobenzene.

0006-2960/93/0432-12949$04.00/0

Scheme I



in Scheme I. That the enzyme is capable of stabilizing σ-complex intermediates is evinced by the fact that the equilibrium constant for σ-complex formation in the active site of isoenzyme 3-3 of GSH transferase (e.g., E·GS⁻ + TNB = E·GSTCD⁻) is about 10^3 greater than it is in aqueous solution [GS⁻(aq) + TNB = GSTCD⁻] (Graminski et al., 1989).

Although the three-dimensional structures of GSH transferases in complex with GSH (Ji et al., 1992) and a variety of other ligands (Reinemer et al., 1991; 1992; Johnson et al.; 1993; Sinning et al., 1993) have been determined, none of the structural details of the interaction with the σ-complex intermediate or the product for this prototypic S_NAr reaction have been elucidated. In this paper we report the first crystal structure of a GSH transferase in complex with the transition state analogue GSTCD⁻ at 1.9-Å resolution and the structure of the enzyme in complex with the product GSDNB at a resolution of 2.0 Å. The two structures represent snapshots along the reaction coordinate for the enzyme-catalyzed S_NAr reaction and reveal specific interactions between the enzyme, intermediate, and product that are important in catalysis.

EXPERIMENTAL PROCEDURES

Crystal Preparation. Crystals were grown from ammonium sulfate solution at pH 8 in the presence of (9*R*,10*R*)-9-(*S*-glutathionyl)-10-hydroxy-9,10-dihydrophenanthrene [(9*R*,-10*R*)-GSPhen)] essentially as previously described (Sesay et al., 1987; Ji et al., 1992) and soaked at room temperature for 6–7 days in a solution of 2 mM GSH and 0.4 mM TNB dissolved in 70% saturated $(NH_4)_2SO_4$ buffered at pH 8 with 25 mM Tris. The soaking solution (ca. 100 μL/crystal) was refreshed six times daily. This procedure was more than sufficient to replace all of the (9*R*,10*R*)-GSPhen originally in the active site. The product complex was formed in a similar manner by soaking crystals in a 100 μM solution of GSDNB. The identical product complex could also be obtained by reacting the crystalline E·GSH complex with CDNB, indicating that the crystalline enzyme is catalytically active.

Data Collection, Structure Solution, and Refinement. Data were collected using a Siemens electronic area detector and Rigaku rotating anode X-ray source as previously described (Ji et al., 1992).[2] Visual inspection of the crystal of the enzyme in complex with GSTCD⁻ indicated that the σ-complex slowly decomposed in the X-ray beam. Therefore, after the first 180° scan in Ω, a new crystal was used for the second orientation. No decomposition of the product complex (E·GSDNB) was noted during data collection. The structures were solved using the 2.2-Å structure of the binary complex of isoenzyme 3-3 and GSH (Ji et al., 1992) as the starting model and refined using the restrained least-squares refinement procedure of Hendrickson (1985) as implemented by Furey et al. (1982). The initial geometry of the trinitrocyclohexadienate anion portion of the σ-complex was based on the crystal structure of 1-methoxy-2,4,6-trinitrocyclohexadienate potassium salt (Destro et al., 1979). A summary of the final refinement statistics is given in Table I.

RESULTS AND DISCUSSION

The GSTCD⁻ anion is found to accumulate in single crystals of isoenzyme 3-3 when soaked in a solution containing GSH and TNB (Graminski et al., 1989), a fact that is readily apparent from the deep red-orange color that the crystals develop as illustrated in Figure 1. Similarly, crystals soaked in the presence of GSDNB turn bright yellow in color. The crystals in which the active site is occupied with the GSDNB show signs of mechanical stress through cracking. However, the crystals were mechanically robust and showed no obvious sign of disorder in the diffraction patterns. The solution and refinement of the two structures were straightforward. The slightly higher crystallographic *R* factors for the refined structures of the two complexes as compared to the E·GSH complex (Ji et al., 1992) are due to some disorder (weak electron density) in the $\alpha4/\alpha5$ helix–turn–helix and the C-terminal tail and are probably related to stress in the crystal lattice manifest in the cracking of the crystals. The disorder is more apparent in subunit B. Nevertheless, the electron density in the active site regions of both subunits in both structures is quite clear.

The $2F_o - F_c$ electron density map for the enzyme in complex with the intermediate analogue GSTCD⁻ is shown in Figure 2a. Interactions between the peptidyl portion of GSTCD⁻ and the protein are identical to those observed in the structure of the E·GS⁻ complex (Ji et al., 1992). The trinitrocyclohexadienate moiety fits snugly into the binding pocket defined by the side chains of Tyr6, Trp7, Leu12, Ile111, Tyr115, Phe208, and Ser209. Of the three nitro groups, two participate in multiple hydrogen-bonding interactions with the protein and associated solvent. The two oxygen atoms of the *p*-nitro group are involved in a bifurcated hydrogen bond with a single water molecule (O1, Figures 2 and 3) which is an integral part of the binding pocket. This water molecule is held in place by interactions with the side chain of Arg107 and Gln165 and is a conserved feature in the active site of isoenzyme 3-3. The hydroxyl groups of Tyr6 and Tyr115 converge on the *pro-R o*-nitro group, each within hydrogen bonding distance of opposite oxygen atoms. One oxygen of the other *o*-nitro group appears to share a single hydrogen bond with a water molecule (O4, Figure 3). Further electrostatic stabilization of the σ-complex may derive from the proximity (4.5 Å) of the *p*-nitro group to the guanidinium cation of Arg107 and the dipole of the $\alpha1$ helix.

The geometry of the σ-complex deserves some comment particularly since it represents the first crystal structure of a σ-complex with a thiolate nucleophile. Carbon 1 of the trinitrocyclohexadienate moiety is sp^3 hybridized with C–C–S bond angles of 105–106°. The structure of the complex differs somewhat from the small-molecule crystal structures of oxygen adducts of 1,3,5-trinitrobenzene, most notably with respect to the orientation of the *o*-nitro groups. Both of the *o*-nitro groups of enzyme-bound GSTCD⁻ are twisted, in opposite directions, about 35° out of the plane of conjugation with the adjacent carbon-carbon double bonds, in contrast to the in-plane

[2] Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument, or equipment identified is the best available for the purpose.

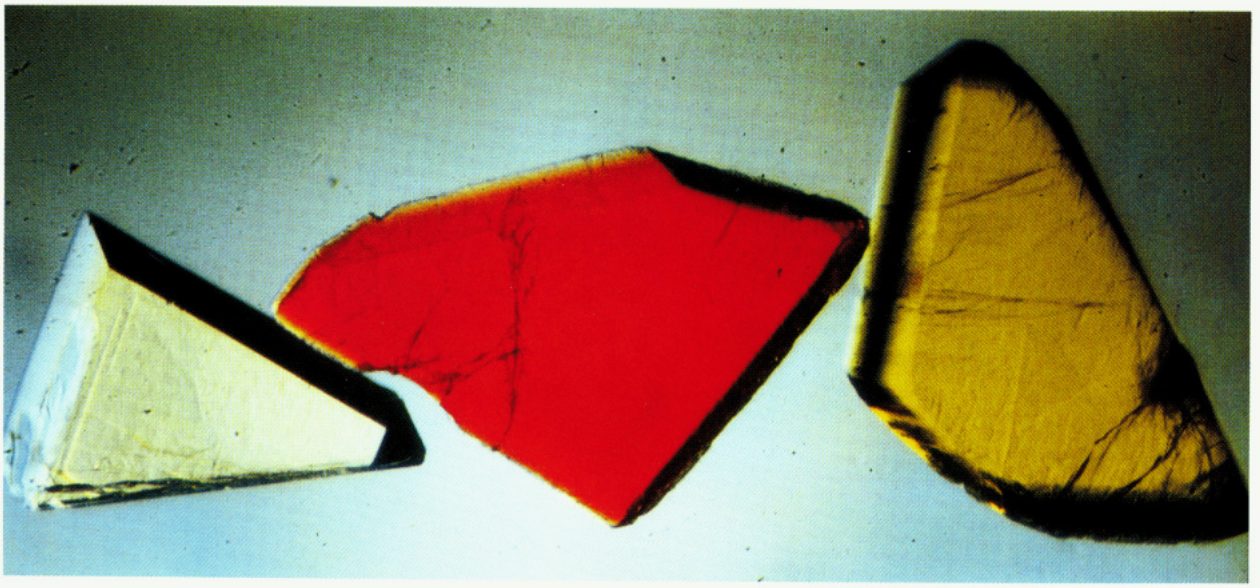


FIGURE 1: Crystals of the substrate, intermediate, and product complexes of isoenzyme 3-3 of GSH transferase. The complexes of enzyme with GSH (colorless), GSTCD$^-$ (red), and GSDNB (yellow) appear from left to right as they would occur on the reaction coordinate.

Table I: Summary of Data Collection and Least-Squares Refinement Parameters

complex	E·GSTCD$^-$	E·GSDNB
D_{min} (Å)	1.9	2.0
total data collected	91596	112537
unique data collected	36138	32038
R_w[a]	8.02	7.32
R_{uw}[b]	6.57	6.64
resolution range (Å)[c]	6.0–1.9	6.0–2.0
reflections used with $I \geq 2\sigma(I)$	29072	24929
crystallographic R factor[d]	0.180	0.194
rms deviations from ideal distances (Å)		
bond distances	0.016	0.019
bond angles	0.040	0.031
planar 1–4 distances	0.036	0.037
rms deviations from ideal chirality (Å^3)	0.211	0.236
thermal parameter correlation (mean/$\Delta\beta$)		
main-chain bond	0.812	1.586
main-chain angle	1.367	2.230
side-chain bond	1.320	1.662
side-chain angle	2.002	2.429

[a] The weighted least-squares R factor on intensity for symmetry-related observations: $R_w = \sum[(I_{ij} - G_{ij}\langle I\rangle_j)/\sigma_{ij}]^2/\sum(I_{ij}/\sigma_{ij})^2$, where $G_{ij} = g_i + A_i s_j + B_i s_j^2$; $s = \sin\theta/\lambda$; and g, A, and B are scaling parameters. [b] The unweighted absolute R factor on intensities: $R_{uw} = \sum(I_{ij} - G_{ij}\langle I\rangle_j/\sum|I_{ij}|)$. [c] Data used for least-squares refinement. All data were used in the calculation of electron density maps. [d] The crystallographic $R = \sum_{hkl}||F_o| - |F_c||/\sum_{hkl}|F_o|$.

orientation observed in 1-methoxy-2,4,6-trinitrocyclohexadienate (Destro et al., 1979). The out-of-plane twists of the *o*-nitro groups are probably imposed by a combination of steric interactions with the sulfur and by interactions with the protein. For example, the hydrogen bond interactions with the hydroxyl groups of Tyr6 and Tyr115 appear to favor the out-of-plane orientation of the *pro-R o*-nitro group while the twist of the *pro-S o*-nitro group minimizes an unfavorable steric interaction with the carbonyl oxygen of the γ-glutamyl peptide bond.

The $2F_o - F_c$ electron density map of the active site of subunit A (Figure 2b) reveals an orientation of the dinitrobenzene moiety in the product complex (E·GSDNB) that is very different from GSTCD$^-$. In marked contrast to E·GSTCD$^-$, the aromatic ring is no longer tucked snugly into the active site cavity, but rather it juts out of the active site toward the crevice located between the two subunits, almost as if the product is on its way out of the active site. The active site cavity, which held the intermediate, is occupied by two additional water molecules (O2 and O3, Figures 2b and 3), one of which bridges the hydroxyl groups of Tyr6 and Ser209. A comparison of the side chains in the active site of subunit A reveals other changes as well. For example, the torsion angle N–CA–CB–OG of Ser209 changes from 67° in EGSTCD$^-$ to –53° in E·GSDNB, eliminating the hydrogen bond, Ser209OH–OHTyr115, in the intermediate complex and forming a new hydrogen bond between Ser209OH and O2 (Figure 3). In addition, the CA–CB–CG1–CD1 torsion angle of Ile111 changes from 116° in E·GSTCD$^-$ to 169° in E·GSDNB, reflecting a loss of a steric interaction between this side chain and a nitro group of the intermediate. The dinitrobenzene moiety in subunit B of the product complex occupies essentially the same position as in subunit A except for a rotation about the S–C bond that places the *o*-nitro group on the opposite side of the ring. This difference in rotamer population must be due to very subtle differences in interactions between the products and the two crystallographically distinct active sites. However, the only other notable difference between the two active sites is the conformation of the side chain of Met108. The alternate conformation of Met108 would create an unfavorable steric interaction between the side chain and the *o*-nitro group in subunit B if the product was bound as it is in subunit A.

How realistic is the intermediate analogue? The most obvious differences between the analogue and the actual intermediate are the presence of the extra nitro group and the absence of the chlorine atom. The latter is perhaps the most serious concern since a realistic structure of the intermediate must be able to accommodate the leaving group. The approximate area that would be occupied by the chlorine atom in the actual intermediate (GSCDCD$^-$) is occupied by a water molecule (O4) located near the edge of the crevice between the two subunits in the structure of the analogue (Figure 3). Thus, there is ample room in the structure to accommodate the leaving group which, conveniently, would appear to be poised near the edge of the solvent channel leading from the active site. Access of the leaving group to solvent is an important consideration since effective hydration of the chloride should contribute to the stabilization of the transition

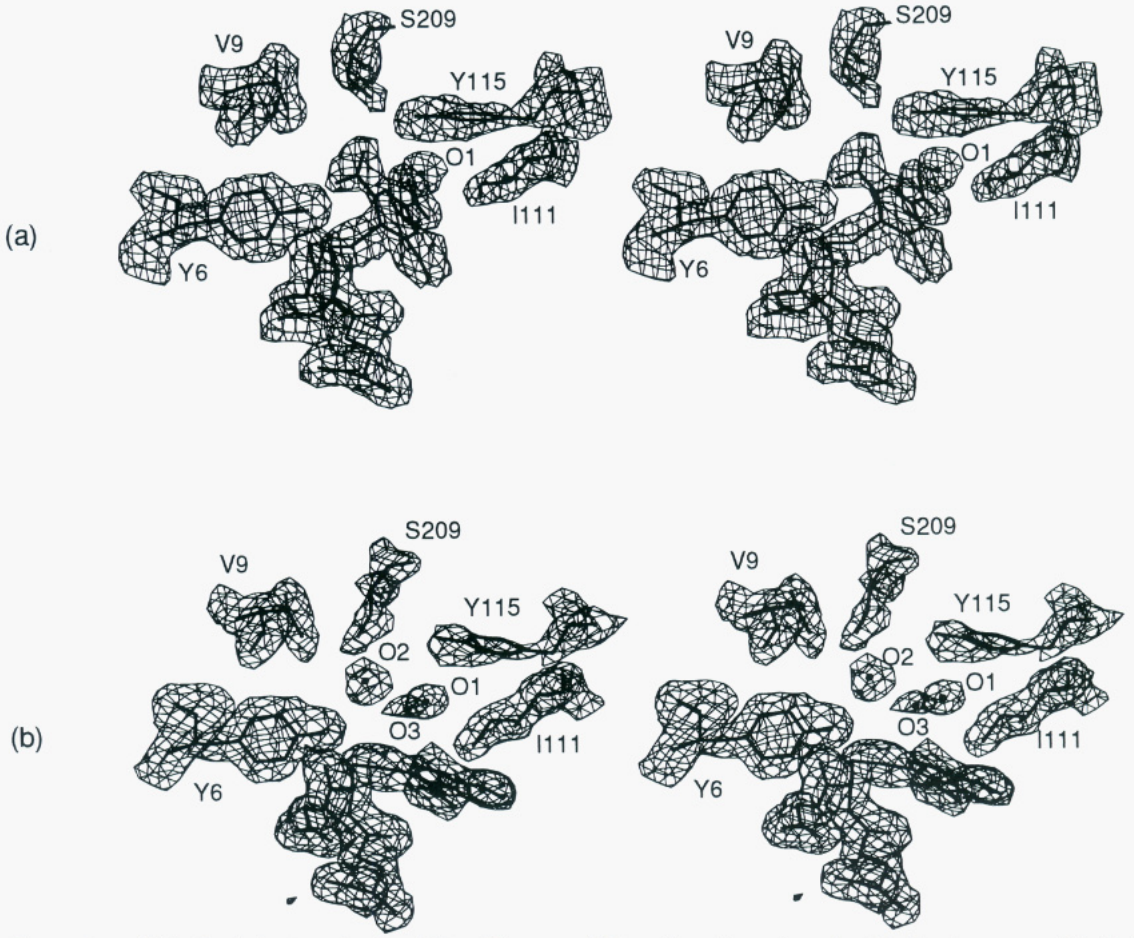


FIGURE 2: Stereoviews of the final electron density ($2F_o - F_c$) maps of the active site contoured at 1σ for isoenzyme 3-3 of glutathione *S*-transferase in complex with (a) the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate anion and (b) 1-(*S*-glutathionyl)-2,4-dinitrobenzene. For clarity, only the products, important protein residues, and water molecules are shown.

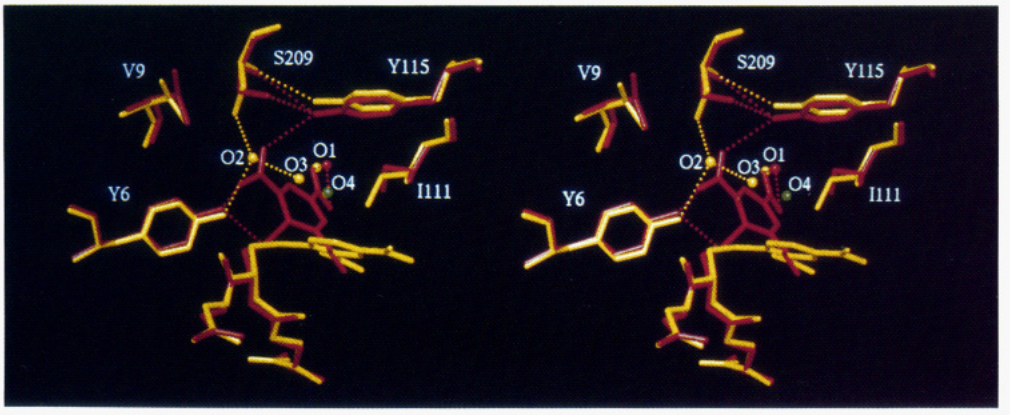


FIGURE 3: RASTER3D (Bacon & Anderson, 1988) representation of the structures of E·GSTCD$^-$ (red) and E·GSDNB (yellow) in subunit A of the dimer. Hydrogen bonds are represented as dotted lines. The water molecule (O4) in E·GSTCD$^-$ that occupies the approximate position of the leaving group (chloride) in the true intermediate GSCDCD$^-$ is shown in green.

state for decomposition of the intermediate.

From the rather different geometries of the enzyme-bound intermediate and product, we postulate reaction coordinate motion for decomposition of the σ-complex intermediate deduced from the crystal structures as illustrated in Figure 4. The absolute configuration of the intermediate at carbon 1 is adduced from the favorable electrostatic interaction that would be provided this diastereomer by the hydrogen bonds between the *o*-nitro group and Tyr6 and Tyr115. The oppositely configured diastereomer would place the *o*-nitro group in an unfavorable steric environment, in close contact with the carbonyl oxygen of the γ-glutamyl peptide bond. Rehybridization of carbon 1 ($sp^3 \rightarrow sp^2$) upon loss of chloride creates an unfavorable steric interaction between the *p*-nitro group and Phe208 in the active site which can be relieved by rotation about the S–C bond (Figure 4). This motion places the aromatic ring of the product out of the active site cavity into the solvent channel between the two subunits. The direction of rotation about the S–C bond cannot, of course, be determined from two pictures. In fact, the observation of an alternative rotamer of the product in subunit B suggests that there is more than one reaction-coordinate motion possible.

It is prudent to mention an important caveat concerning the structures of the intermediate and product complexes. It

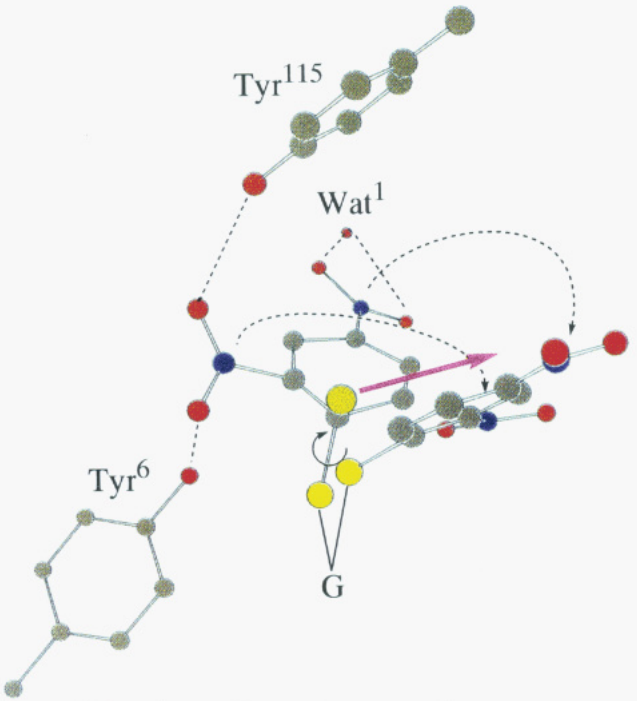


FIGURE 4: Reaction coordinate motion proposed for the decomposition of (1*R*)-1-chloro-1-(*S*-glutathionyl)-2,4-dinitrocyclohexadienate in the active site of subunit A of isoenzyme 3-3. Atoms are colored according to element type: carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; and chlorine, green. Hydrogen-bonding interactions are shown as broken lines. The solid arrow indicates a possible trajectory of the chloride ion toward the solvent channel while the broken arrows show the motion of the nitro groups. The distances traversed by the *o*- and *p*-nitro groups deduced from the subunit A structures are 5.7 and 7.9 Å, respectively. In subunit B, where the *o*-nitro group is on the opposite side of the ring in the product complex, the distances traversed by the two nitro groups are 3.5 and 7.5 Å.

is certainly possible that the crystallographic complexes obtained through diffusion of the materials into a preformed crystal may not accurately represent the most relevant conformations along the reaction coordinate. The very fact that stress in crystals is evident suggests that the lowest energy conformation of the complexes may not be entirely compatible with the crystal lattice. The stress in the crystals indicates that protein motions, which are not observed in the final structures, may occur during the soaking process. This issue might be resolved by cocrystallization of the protein and the relevant ligands. However, the fact remains that the intermediate and product complexes readily form by reaction of GSH with either TNB or CDNB in the crystal. Therefore, it is probable that each structure contains realistic information about the reaction coordinate for the enzyme-catalyzed S_NAr reaction.

What do the structures of the enzyme-bound intermediate and product really tell us about the reaction coordinate? One structure that is obviously missing is the ternary E·GS⁻·CDNB complex that flanks the intermediate on the reaction coordinate (Figure 5). It is likely that the geometry of the productive ternary complex resembles that of the intermediate with the sulfur of GS⁻ approaching the lobe of the π-orbital at carbon 1 of CDNB. We reiterate that the structure of the binary E·GS⁻ (Ji et al., 1992) is virtually identical to that of the E·GSTCD⁻ complex, except for the solvation of the sulfur in the former and the presence of the trinitrocyclohexadienate group in the latter. Thus, very little structural reorganization should be necessary in formation of the intermediate from a productive ternary enzyme–substrate complex. In contrast, there is apparently a great deal of motion subsequent to the

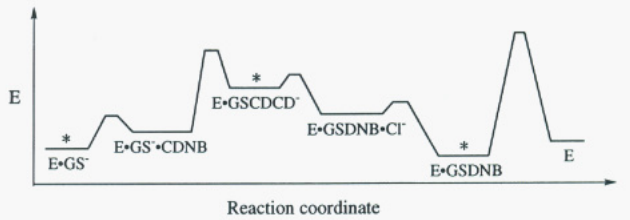


FIGURE 5: Schematic of the reaction coordinate for the enzyme-catalyzed addition of GSH to CDNB. The complexes for which structural information is now available are indicated with asterisks. The relative energies of the various complexes should not be inferred from the diagram.

decomposition of the intermediate. We do not mean to suggest that the crystallographically observed E·GSDNB complex necessarily depicts the conformation of the product complex that *immediately* flanks the σ-complex intermediate on the reaction coordinate (e.g., E·GSDNB·Cl⁻, Figure 5). It is likely that the species next to the intermediate is of higher energy, with the dinitrobenzene moiety still in the active site cavity. The observed product complex is lower in energy and probably represents the ground state for product release, the rate-limiting step in the isoenzyme 3-3-catalyzed addition of GSH to CDNB (Johnson et al., 1993).

Structural information is now available for three of the six species illustrated in the reaction coordinate diagram of Figure 5. Of the three missing structures, one, that of the unliganded enzyme, is probably attainable. The two species that flank the intermediate represent a much more formidable challenge. Although many of the details of the reaction coordinate motion such as the direction of the rotation about the S–C bond or the importance of other conformers in the decomposition of the σ-complex cannot be determined from the two snapshots presented here, these results clearly delineate specific interactions between the enzyme, intermediate, and product important in catalysis and provide realistic starting points for molecular dynamics simulations of the reaction coordinate motion for this reaction.

In summary, the three-dimensional structures of a glutathione transferase in complex with an analogue of the σ-complex intermediate and the product of an S_NAr reaction reveal specific interactions with the protein that are important in catalysis. The σ-complex appears to be stabilized by hydrogen-bonding interactions between the *pro-R* *o*-nitro group and the hydroxyl groups of Tyr6 and Tyr115 and between the *p*-nitro group and an enzyme-bound water. The cationic side chain of Arg107 and the dipole of the α1 helix appear to provide a favorable electrostatic environment for the GSTCD⁻ anion. In contrast, the product, GSDNB, is observed in a completely different orientation with the dinitrophenyl group sticking out of the active site cavity and into the crevice between the two subunits. The results are consistent with a reaction coordinate motion for decomposition of the σ-complex to product that involves movement of the dinitrophenyl group out of the active site subsequent to loss of chloride and the rehybridization of carbon 1.

REFERENCES

Armstrong, R. N. (1991) *Chem. Res. Toxicol. 4*, 131–140.
Armstrong, R. N. (1993) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).
Bacon, D., & Anderson, W. A. (1988) *J. Mol. Graphics 6*, 211–220.
Bernasconi, C. F. (1980) *Chimica 34*, 1–11.
Chen, W.-J., Graminski, G. F., & Armstrong R. N. (1988) *Biochemistry 27*, 647–654.

Clark, A. G., & Sinclair, M. (1988) *Biochem. Pharmacol. 37*, 259–263.
Destro, R., Pilati, T., & Simonetta, M. (1979) *Acta Crystallogr. B35*, 733–736.
Furey, W., Wang, B. C., & Sax, M. (1982) *J. Appl. Crystallogr. 15*, 160–166.
Graminski, G. F., Zhang, P., Sesay, M. A., Ammon, H. L., & Armstrong, R. N. (1989) *Biochemistry 28*, 6252–6258.
Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974) *J. Biol. Chem. 249*, 7130–7139.
Hendrickson, W. (1985) in *Crystallographic Computing 3: Data Collection, Structure Determination, Proteins, and Databases*(Sheldrick, G., Kruger, C., & Goddard, R., Eds.) pp 306–311, Clarendon Press, Oxford.
Ji, X., Zhang, P., Armstrong, R. N., & Gilliland, G. L. (1992) *Biochemistry 31*, 10169–10184.
Johnson, W. W., Liu, S., Ji, X., Gilliland, G. L., & Armstrong, R. N. (1993) *J. Biol. Chem. 268*, 11508–11511.
Miller, J. (1968) in *Reaction Mechanisms in Organic Chemistry* (Eaborn, C., & Chapman, N. B., Eds.) Vol. 8, pp 137–179, Elsevier, New York.
Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O., & Huber, R. (1991) *EMBO J. 10*, 1997–2005.
Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G., & Parker, M. W. (1992) *J. Mol. Biol. 227*, 214–226.
Rushmore, T. H., & Pickett, C. B. (1993) *J. Biol. Chem. 268*, 11475–11478.
Sesay, M. A., Ammon, H. L., & Armstrong, R. N. (1987) *J. Mol. Biol. 197*, 377–378.
Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B., & Jones, T. A. (1993) *J. Mol. Biol. 232*, 192–212.