Spectral Differences between Rhodanese Catalytic Intermediates Unrelated to Enzyme Conformation*

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Circular dichroism (CD) spectra and UV absorption spectra of two obligatory intermediates in rhodanese catalysis were compared. A broad CD band between 250 and 287 nm increased in a manner stoichiometrically related to the content of enzyme-bound persulfide. Titration of a sample of sulfur-substituted rhodanese (ES) with either cyanide or sulfite gave a stoichiometry that is consistent with one persulfide/molecule of rhodanese ($M_0 = 33,000$). This result agrees with that determined by x-ray crystallography and a method based on quenching of intrinsic fluorescence. Cyanalysis of the persulfide in ES is accompanied by a decrease of UV absorption in the region between 250 and 300 nm. Cyanide titrations followed by the change in absorbance at 283, 272, and 292 nm gave the expected stoichiometry. The magnitude of the difference between the far UV-CD spectra of $E$ and ES found here is smaller than reported previously. This variability suggests that the differences in the secondary structure of these intermediates may not be obligatorily related to the cyanalysis of the persulfide. This view is compatible with recent evidence which suggested that $E$ and ES may be made different by structural relaxation events that occur outside of the catalytic cycle. Furthermore, the methods developed here will be useful in studies on the stability of the catalytic persulfide that has been suggested to be central in the mechanism of several enzymes important in sulfur metabolism.

Rhodanese, thought to be an important regulator of sulfur flux through a cell's sulfane pool (1, 2), can catalyze the transfer of sulfur from thiocyanate to cyanide by a double displacement mechanism in vitro (Reactions 1 and 2). The single polypeptide of 293 amino acids can be isolated as either the sulfur-free enzyme ($E'$) and the sulfur-substituted form (ES). The transferable sulfur atom in ES is bound to the active site sulfhydryl in a persulfide bond (3-5).

\[ E + S_2O_7^{2-} \rightleftharpoons ES + SO_4^{2-} \tag{1} \]
\[ ES + CN^- \rightarrow E + SCN^- \tag{2} \]

Much effort has been focused on deducing an expanded catalytic mechanism that includes putative enzyme conformational changes. Based on the pioneering work of Volini and Wang (6, 7), it has been proposed that $E$ and ES are structurally different in solution; and subsequent studies that have compared the physical properties of these intermediates gave results that are compatible with that proposal (8-10). Other kinetic studies suggested that conformational malleability of this enzyme may be the basis for the anomalous kinetic behavior observed (11, 12). The solution results seemed to indicate that the enzyme can cycle between the two conformers. In contrast to the solution studies, x-ray crystallographic studies have failed to see significant conformational differences between the two intermediates in a crystalline environment (13, 14). This observation is incompatible with the idea that enzyme structural changes are catalytically coupled. There is, as yet, little supporting data for explanations of the observed discrepancies between the crystallographic and solution studies (15).

It is generally accepted that $E$ and ES are conformationally distinct in solution; however, the reported magnitude of the difference as indicated by CD and UV absorption spectral differences between the two forms has varied. A comparison of the data from the report that first documented spectral differences between $E$ and ES (6) with the data of a more recent study (16) shows intriguing differences. Understanding these discrepancies is important because spectral differences between $E$ and ES are taken to be reflections of conformational differences between these catalytic intermediates. Furthermore, the magnitude of the structural differences places constraints on the appropriateness of the models used to understand the functional behavior of rhodanese.

In this report, we have examined the difference UV absorption spectra and CD spectra of $E$ and ES. The results suggest that the magnitude of the conformational differences between the two catalytic intermediates may be more subtle than previously suggested and those differences may not be obligatorily coupled to catalysis. This view is consistent with the recent evidence suggesting that differences between $E$ and ES may develop in processes that occur outside of the catalytic cycle (12).

**EXPERIMENTAL PROCEDURES**

All reagents used were analytical grade. Bovine liver rhodanese was prepared as previously described (17) and was stored at $-70 \, ^\circ C$ as a crystalline suspension of ES in 1.8 M ammonium sulfate containing 1 mM sodium thiosulfate. Stock solutions of the ES form were prepared by washing crystalline ES twice with 1.8 M ammonium sulfate to remove the excess thiosulfate. The washed pellet was dissolved to a protein concentration of 40-50 mg/ml with 10 mM sodium phosphate buffer, pH 7.0. Enzyme activity was measured by a colorimetric method based on the quantitation of the product thiocyanate as a ferric thiocyanate complex (18). The protein concentration was determined spectrophotometrically using $E_{\text{max}}^{1%} = 1.75$ (19). Unless otherwise stated, experiments were performed in buffer containing 50 mM Tris-SO$_4$, pH 8.0.

Circular dichroism measurements were performed in a Jasco Model...
Spectral Differences between Rhodanese Intermediates

Current interest in the occurrence of conformational changes in rhodanese during catalysis makes it important to resolve the discrepancies between the reports of the CD and UV absorption spectra of two rhodanese catalytic intermediates. Volini and Wang (6) have reported no significant differences in the near UV-CD spectra of E and ES while Cannella et al. (16) have reported a positive band between 250 and 310 nm in the spectrum of E that is not in ES (see Table I). It was postulated that this broad band could be due to the perturbation of aromatic side chains and that the band between 255 and 277 nm could be due to the persulfide group. However, no direct correlation between the intensity of the CD band with the content of enzyme-bound sulfur was shown. If this band indeed reflects the content or the environment of the persulfide, then its intensity should be related stoichiometrically to the removal of the persulfide from ES. The spectra shown in Fig. 1 are similar to those reported by Cannella et al. (16, 20) in shape and magnitude. The local maximum at 265 nm is about 26 degrees cm$^2$ dmol$^{-1}$, and about 64 degrees cm$^2$ dmol$^{-1}$ at 292 nm. The addition of KCN to a sample of ES is accompanied by an increase of the ellipticity of a broad band between 260 and 287 nm. The peak around 292 nm that has been attributed to tryptophanyl side chains is not significantly changed with KCN additions (Fig. 1). The per cent change in ellipticity at 263 nm is plotted as a function of the ratio of cyanide to enzyme (Fig. 2a). The equivalence point is reached at the cyanide to enzyme ratio of 0.89. This value is consistent with the expected stoichiometry of 1 cyanolyzable persulfide/molecule of rhodanese ($M_r = 33,000$). Furthermore, as implied by Reaction 2, cyanolysis of the persulfide in ES has been reported to be irreversible and to include no kinetically significant complex (21, 22). This is reflected by the sharp break in the titration curve once the expected stoichiometry has been reached.

A similar titration performed using sodium sulfite as the titrant also gives the expected stoichiometry. The equivalence point is reached at a sulfite to enzyme ratio of 1.1. The reversibility of Reaction 1 apparently is reflected in the shape of the titration curve shown in Fig. 2b as contrasted with that shown in Fig. 2a. Likewise, addition of the donor substrate thiosulfate which reforms the persulfide conforms to the equilibrium process (about 75% restored at a ratio of [thiosulfate]/[enzyme] = 30). The data shown in Fig. 2b fit reasonably well with those reported in the intrinsic fluorescence quenching studies (23) as shown by the open circles.

Specific activity measurements indicate that the enzyme is fully active at the end of the titration. Neither the addition of NaCl nor Na$_2$SO$_4$ appears to affect the ellipticity in a comparable manner, so nonspecific anion-induced conformational changes can be discounted. The close correlation of the CD change with the addition of substrates that lead to reactions as predicted by Reactions 1 and 2 suggests that the titration of enzyme-bound persulfide is being monitored.

The previous reports further disagreed in the difference UV

### Table I

| $[\theta]_{520}$ (degrees cm$^2$ dmol$^{-1}$) | $\Delta[\theta]_{520}$ (degrees cm$^2$ dmol$^{-1}$) |
|------------------------------------------|-----------------------------------------------|
| This report                              | 10                                           |
| Ref. 6                                   | 15                                           |
| Ref. 60                                  | 23                                           |
| Ref. 20                                  | 23                                           |

$\Delta[\theta]_{520} = [\theta]_{ES} - [\theta]_{ES0}$

$[\theta]_{ES0}$ is the ellipticity of the sulfur-free rhodanese and $[\theta]_{ES}$ is that of the sulfur-substituted form. An analogous expression was used for $\Delta[\theta]_{292}$. The ES sample contained 38 $\mu$M ES, 50 mM Tris-SO$_4$, pH 8.6, 23 °C; the E sample included KCN at 55 $\mu$M. The E sample contained 39 $\mu$M enzyme, 10 mM Tris-SO$_4$, pH 8.6, 27 °C, 0.2 mM SO$_4^2^−$, 0.2 mM S$_2$O$_3^2$; the ES sample contained: 39 $\mu$M enzyme, 10 mM Tris-SO$_4$, pH 8.6, 27 °C, 0.2 mM SO$_4^2$; 1 $\mu$M SO$_2$; 340 $\mu$M S$_2$O$_3$. The ES form was converted to E with either CN$^−$ or SO$_4^2 -$.

The ES sample contained 34 $\mu$M enzyme, 10 mM phosphate buffer, pH 7.6, 10 °C. The E sample also contained either 62 $\mu$M CN or 248 $\mu$M SO$_4^2$.

The previous reports further disagreed in the difference UV
absorption spectrum of E and ES in the region between 260 and 300 nm. The relationship of the CD difference in the aromatic region to the persulfide suggested that an absorption difference in the same region may also be related to the persulfide. Fig. 3a shows a difference spectrum of the two intermediates. Titration of an ES sample with KCN was performed by monitoring the difference absorption change. Changes of intensity at 263, 272, and 292 nm were plotted as a function of the ratio [cyanide]/[enzyme] (Fig. 3b). The plots of the fraction of the difference change monitored at 263 nm and 292 nm are analogous to that shown in Fig. 2a and gave the expected stoichiometry. The plot of the change at 272 nm showed scatter that could be a reflection of the noise in this region (Fig. 3a). The stoichiometry determined by this method is the same as that determined when the near UV-CD change is monitored.

Several reported CD spectra of E and ES in the peptide backbone region differed in magnitude (see Table II). Fig. 4 shows the far UV-CD spectra of E and ES. Although the overall shapes of the CD spectra are comparable, the magnitude of the difference between the spectra of E and ES is about one-third of that reported previously. The ellipticity of ES at 220 nm (about -6000 degrees cm² dmol⁻¹) agrees with the value reported previously; however, the ellipticity of E at the same wavelength (about -6400 degrees cm² dmol⁻¹) is significantly less. Since the magnitude of the difference in the far UV-CD spectra is taken to indicate the extent of secondary structural differences, this result leads one to suspect that the conformational differences between E and ES may be more subtle than previously suggested. Furthermore, the stoichiometric relationship between the magnitude of this CD difference and enzyme-bound sulfur has not been shown; so, the question of whether the presumptive structural differences are obligatory to the interconversion between the two intermediates remains open.

In view of the evidence showing conformational changes in E in the absence of sulfur donors (7) and the more recent evidence suggesting that the conformation of the enzyme is determined by a number of competing processes (12), the observed discrepancies may have bases related to the functional behavior of the enzyme. One factor that could have contributed to the inconsistencies observed is that composition of an enzyme sample thought to be ES may contain some E forms. It has been pointed out that sulfur can dissociate from the enzyme during recrystallization and other purification procedures (24). Different solution methods used to quantitate the bound sulfur have reported a range of stoichiometries (4, 25, 26). This uncertainty may reflect the inherent instability of the persulfide bond. The ability to quantitate and monitor the enzyme-bound persulfide is particularly important because of its central role in the catalytic mechanism.

The various methods designed to quantitate the persulfide have been unsatisfactory. One based on the occurrence of a weak absorption band (E = 80 cm⁻¹ m⁻¹ at 330 nm (4)) in ES required conditions [enzyme] = 60 mg/ml too restrictive to be generally useful in studies on the functional relevance of enzyme conformational change; furthermore, no stoichiometric relationship between this band's intensity and sulfur content of the enzyme has been established. A more sensitive method is based on the discovery that the intrinsic fluorescence of ES is about 30% lower than that of E (17). Although this method gives the accepted stoichiometry and equilibrium constant for Reaction 1, the quenching mechanism is unknown and may involve processes that are not directly related to the presence of the persulfide (12).

A more interesting possibility is that the different conditions used may have contributed to the variability observed in the far UV-CD spectra of rhodanese. In the earlier study, the buffer contained a delicate balance of thiocylate, sulfite, and sulfate (6). As implied by Reaction 1, a mobile equilibrium exists between E, ES, and (E S₂O₄⁻) under these conditions such that the spectrum reflects contributions from each of these species. In the more recent study, the spectrum of E was obtained after an ES sample was treated with a 2-fold molar excess of cyanide. In light of the evidence of E conformational autoconversions, it is questioned whether the far UV-CD spectrum reported for E is in fact an average of all the sulfur-free conformers that existed in solution under these conditions. Variations in the populations of sulfur-free conformers in the sample could account for the variability in the magnitudes reported.

It is important to note that it has not been established that the differences in the near UV-CD and UV absorption reflect conformational differences between the two intermediates.
Fig. 3. Difference absorption spectrum E and ES. Spectral scans were performed in a Cary 219 spectrophotometer. The base-line was established by scanning the sample cuvette containing 29 μM of ES, 50 mM Tris·SO₄, pH 8.6, against the reference cuvette containing an identical solution. To obtain the difference spectrum, microliter quantities of a KCN solution made in 50 mM Tris·SO₄, pH 8.6, was added to the sample cuvette to make the sample 50 μM in KCN. The same volume of buffer without KCN was added to the reference cuvette. b, fraction of absorption change as a function of KCN concentration. The experiment was set up as described above. KCN was added to the sample cuvette in 5-μl increments. With each KCN addition, the same volume of buffer without KCN was added to the reference cuvette. ΔA = A_obse/A_obse = the absorbance observed at 0, 263 nm; O, 272 nm; or A, 292 nm after each KCN addition. AE = the maximum difference at these wavelengths. The equivalence point is reached at 1 KCN/molecule of enzyme.

Table II
Comparison of far UV-CD ellipticities of E and ES

|       | [θ]_222° | [θ]_208° | Δ[θ]* |
|-------|----------|----------|-------|
| This report* | -6400 | -6000 | 400    |
| Ref. 6* | -7500 | -6200 | 1300   |
| CN⁻  | -7100 | -6400 | 700    |
| SO₄⁻  | -6800 | -6400 | 400    |

* As in Table I.
* Buffer contained: 4.2 μM enzyme, 10 mM Tris·SO₄, pH 8.6, 23 °C without (ES), or with (E), 3 mM KCN.
* Buffer contained: 30 μM enzyme, 10 mM Tris·SO₄, pH 8.6, 27 °C. The ES sample also contained: 0.2 mM SO₄⁻, 1 μM SO₄⁻, 340 μM S₂O₄²⁻. The E sample also contained: 0.2 mM SO₄⁻, 0.2 mM SO₄⁻, 10 μM S₂O₄²⁻.
* Buffer contained: 14 μM enzyme, 10 mM phosphate buffer, pH 7.6, 10 °C. E was prepared with either CN⁻ (28 μM) or SO₄⁻ (112 μM).

The absorption band in ES between 250 and 300 nm is apparently related to the presence of the persulfide. First, Villarejo and Westley (27) have shown that organic persulfides absorb in this region. Second, the absorption spectrum of alkanal-treated insulin contain two local maxima (240 and 323 nm) that are related to cyanolyzable protein-bound persulfide (28). Third, Cannella et al. have attributed the absorbance at 276 nm and 335 nm to the persulfide group (16). Fourth, it is unlikely that the absorption band between 250 and 280 nm is related to disulfides because none have been found in the fully active rhodanese (29, 30). Finally, the correlation with the negative CD band in the same wavelength region and the relationship to the stoichiometry described argue that this absorption difference reflects the content of enzyme-bound persulfide.

Fig. 4. Far UV-CD spectra of E and ES. Spectra were obtained in a Jasco J-500 spectropolarimeter with data processing accessory. Samples containing 4.2 μM enzyme, 10 mM Tris·SO₄, pH 8.6, with (— — —) or without (— — —) 3 mM KCN were scanned in a cylindrical quartz cuvette of 0.1-cm path length at 23 °C. The difference between the spectra of E and ES at 220 nm was about 360 degrees cm² dmol⁻¹.

However, the broad negative band from 250 to 300 nm also overlaps the absorption region of tryptophans, tyrosines, and phenylalanines. It is possible that sulfur removal from the active site perturbs the aromatic side chains, possibly those that make up the hydrophobic cluster (31) implicated in
stabilizing the persulfide. This would be consistent with the 
suggestion that the conversion is associated with conforma-
tional change. Since the relative contribution to the observed 
asorption difference by the persulfide and the perturbation of 
the aromatic side chains is unknown, the extent of confor-
mational change remains an open question.

In conclusion, the negative CD band between 255 and 270 
nm appears stoichiometrically related to the persulfide within 
the rhodanese active site. These findings provide a basis for 
a facile procedure which quantitates and monitors the en-
zyme-bound persulfide. In addition, these methods may be 
generally useful to study the properties of enzyme-bound 
 persulfides (32-34). More importantly, the assignment of this 
band to the persulfide explains part of the near UV-CD 
spectral differences found between E and ES. Subtle struc-
tural differences are also indicated. Likewise, the UV absorp-
tion difference found between the two intermediates reflects 
the presence of the persulfide in ES and may also indicate 
small conformational differences between the two forms. The 
results reported here are compatible with the recent evidence 
suggesting that some conformational differences between E 
and ES depend on processes that take the enzyme outside of 
of the catalytic cycle (12). One might speculate that although E 
and ES have distinct conformational potentialities, their 
structures during catalysis may be more similar than previ-
ously thought. This view would accommodate the observation 
that there is no significant conformational change associated 
with the cyanolysis of the persulfide in the rhodanese crystal.

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