Active Site Modifications Quench Intrinsic Fluorescence of Rhodanese by Different Mechanisms†

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ABSTRACT: Beef liver rhodanese can be modified covalently at the active site (Cys-247) either reversibly or irreversibly by sulfur, selenium, iodoacetate, and hydrogen peroxide. Each derivative shows an intrinsic fluorescence lower than that of the free enzyme. The reaction of rhodanese with iodoacetate or hydrogen peroxide is time-dependent and accompanied by enzyme inactivation, by the loss of one or two sulfhydryl groups, respectively, by quenching and bathochromic shift of fluorescence, and by an absorbance perturbation in the near UV. The latter findings are indicative for a displacement of some tryptophyl side chains from hydrophobic to hydrophilic environment. The fluorescence decays of the various rhodanese derivatives can be fitted by a double-exponential function with two lifetimes: a shorter one of 1–1.7 ns and a longer one of 2.8–4.6 ns. The S-loaded and Se-loaded rhodanese samples have proportionally shorter lifetimes and lower quantum yields. No such proportionality was observed for the iodoacetate-treated and for the hydrogen peroxide treated enzyme. These findings indicate that two different quenching mechanisms are operating in rhodanese derivatives, a long-range energy transfer from tryptophan to persulfide (or sulfoselenide) group and a static quenching accompanying a conformational change of the protein after modification of the active site.

Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) is an ubiquitous protein showing a sulfurtransferase activity of yet unclear physiological meaning [for reviews, see Sörbo (1975) and Westley (1973)]. In vitro it catalyzes the transfer of the outer sulfur atom from thiosulfate to cyanide. In the course of its activity, rhodanese cycles between two catalytic intermediates, i.e., a sulfur-loaded enzyme, ES, and a sulfur-free enzyme, E. In the ES form the transferred sulfur is bound to Cys-247 as a persulfide group (E–S–SH), which shows a weak absorbance at 270 and 335 nm (Cannella et al., 1980; Finazzi-Agrò et al., 1972). Similarly, rhodanese in the E form can take selenium from selenosulfate with the formation of a sulfoselenide derivative (E–S–SeH) absorbing at higher wavelengths. The intrinsic fluorescence of rhodanese is quenched upon binding of sulfur or selenium. It has been proposed that this quenching results from energy transfer between the excited tryptophan residues and the chromophoric group (Cannella et al., 1975; Finazzi-Agrò et al., 1972). X-ray structure and biochemical studies indicate that the active site is located close to a cleft in between the two domains formed by the single polypeptide chain and that virtually all side chains essential for the catalysis are provided by the second domain only (Ploegman et al., 1978a). There are reports suggesting that X-ray crystallography does not fully reflect the dynamics of the protein in solution (Chow et al., 1985). In particular, tritium exchange experiments seem to indicate a different conformation of E and ES forms of rhodanese (Horowitz & Falksen, 1983).

The active site sulfhydryl group is unreactive in the ES or ESe forms. The removal of sulfur or selenium makes the essential sulfhydryl group available to alkylation by iodoacetate or to oxidation by hydrogen peroxide (De Toma & Westley, 1970; Cannella & Berni, 1983). In the latter case, it seems to form an intramolecular disulfide bond with a second sulfhydryl group close to it in the active site. The fluorescence of carboxymethylated and oxidized derivatives of rhodanese is quenched more or less as the sulfur-containing form. Fluorescence offers a very useful tool for the study of protein at catalytic concentrations. Furthermore, the fluorescence lifetimes are in the time scale of protein mobility. These considerations induced us to reinvestigate the mechanism of fluorescence quenching in rhodanese by means of static and dynamic fluorescence.

Experimental Procedures

Selenosulfate was prepared according to Klebanov and Ostapkevic (1962). Iodoacetic acid was recrystallized from carbon tetrachloride; the solution of iodoacetic acid was neutralized with NaOH before use. Urea was "special quality for electrophoresis" from Bio-Rad; sodium thiosulfate "suprapur" was from Merck; all other chemicals were analytical-grade.

Bovine liver rhodanese was prepared according to the procedure of Horowitz and De Toma (1970) and was twice crystallized. Protein concentration and catalytic activity was determined according to Sörbo (1953). The enzyme is homogeneous by ultracentrifuge and SDS–polyacrylamide gel electrophoresis and has a specific activity of 550 units/mg. One enzyme unit is defined as the amount of protein converting 1 μmol of cyanide to thiocyanate per minute (pH 8.65, 20 °C). The enzyme was stored, in the sulfur-containing form, at 4 °C as a microcrystalline suspension in 1.8 M ammonium sulfate, containing 1 mM thiosulfate, brought to pH 7.8 with ammonia. Before use, an aliquot of this suspension was centrifuged; then the precipitate was dissolved in 50 mM phos-

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phate buffer, pH 7.6, and dialyzed overnight against the same buffer. Sulfur-free enzyme was prepared by adding a stoichiometric amount of cyanide to the dialyzed sulfur-containing form. Rhodanese derivatives were obtained by incubating the free enzyme in 50 mM phosphate buffer, pH 7.6, with selenosulfate as previously reported (Cannella et al., 1975), with an equimolar amount of hydrogen peroxide (Cannella & Berni, 1983), or with a small molar excess (2 equiv) of iodoacetate. When necessary, the excess of reagents was removed from the incubation mixture by gel filtration on a Sephadex G-25 column. Sulfhydryl content of rhodanese was assayed by reaction with Ellman's reagent in the presence of 8 M urea (Pensa et al., 1977).

Fluorescence spectra were recorded on a Perkin-Elmer MPF3 spectrofluorometer. Absorption spectra were measured on a Beckman 5260 or DU8 spectrophotometer equipped with thermostated cell holders. Fluorescence lifetimes were measured with the single photon counting technique by using an instrumental apparatus assembled in our laboratory with commercial components. The pulsed lamp is the Model 199F from Edinburgh Instruments (U.K.). The excitation and emission optics, sample compartment, and photomultiplier are produced by Applied Photophysics (U.K.). Amplifiers, constant-fraction discriminators, and time-to-amplitude converter are from Ortec. The multichannel analyzer is the Model 7934 from Silena (Italy). Decay data were analyzed with a least-squares iterative reconvolution to correct for the time profile of the excitation pulse (Grinvald & Steinberg, 1974) running on a PDP-11-23 Digital computer. Samples were excited at 280 nm (10-nm bandwidth) with an Applied Photophysics monochromator, and fluorescent light was collected after passage through a solution of Triton X-100 (OD = 2 at 285 nm) to filter off the scattered light. All the rhodanese derivatives had an optical density at 280 nm of about 0.6. The time profile of the excitation pulse was recorded at 280 nm with an aluminum reflector. The rate of photon counting (start/stop ratio) was always lower than 0.02 to avoid pile-up distortions.

Results

Modification of the active site cysteine of beef liver rhodanese is accompanied by a quenching of the intrinsic protein fluorescence. Figure 1 shows such effect for the sulfur-loaded enzyme (curve A′) and for the carboxymethylated enzyme (curve C′). Both derivatives are less fluorescent than the sulfur-free form (curve B′). However, subtle spectral differences between the two are evident. Carboxymethylated rhodanese has an emission spectrum with a maximum 5-nm shift toward the red with respect to the sulfur-containing and to the sulfur-free enzyme. The selenium-loaded rhodanese has a fluorescence emission very similar to that of sulfur-containing enzyme but an even lower quantum yield (Cannella et al., 1975). Figure 1 also shows the difference absorption spectra in the region 370–240 nm generated by derivatization of rhodanese. The unloading of sulfur from rhodanese with cyanide is accompanied by a decrease in the absorbance with minima at 335 and 270 nm (curve B). The latter through shows also ripples, which become prominent peaks at 292 and 285 nm when sulfur-free enzyme is reacted with iodoacetate (curve C). Furthermore, several fine structure features appear at 281, 275, and 271 nm, typical of tryptophan perturbation (Strickland et al., 1970). A maximum perturbation with $\Delta\epsilon_{292}$ = 2700 M^{-1} cm^{-1} was obtained, after 30-min incubation of sulfur-free rhodanese with a small excess (2 equiv) of iodoacetate. A more rapid reaction leading to the same final effect was observed at higher iodoacetate concentrations.

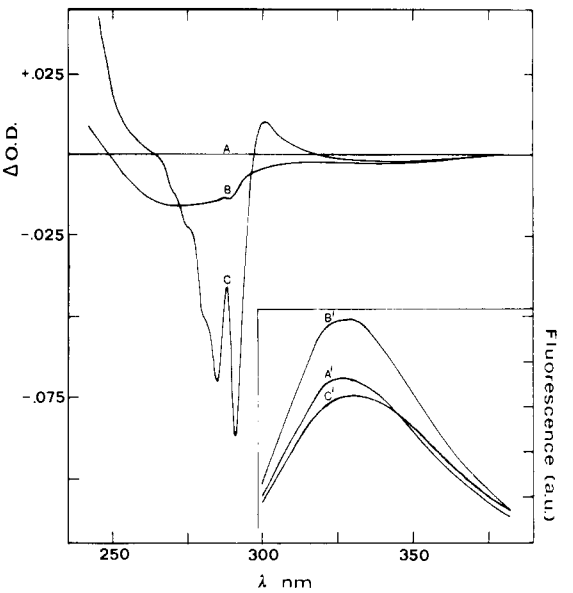


FIGURE 1: Spectroscopic changes induced by carboxymethylation of rhodanese. Difference absorption spectra. (Curve A = base line) both sample and reference cuvettes contained 26 μM sulfur-loaded enzyme (ES) in 50 mM phosphate buffer, pH 7.6. (Curve B = E vs. ES) Upon addition of 30 μM cyanide to the sample. (Curve C = E carboxymethylated vs. ES) spectrum was taken 30 min after treating the sulfur-free enzyme with 50 μM iodoacetate. Reagents were added to the sample in microliter amounts and a corresponding amount of buffer was added to the reference. Fluorescence spectra. Curves A′, B′, and C′ correspond to the samples A, B, and C described above upon 1:10 dilution with buffer. Excitation wavelength was 280 nm; excitation and emission slit was 10 nm. All spectra were monitored at 20 °C in 1-cm light-path cuvettes.

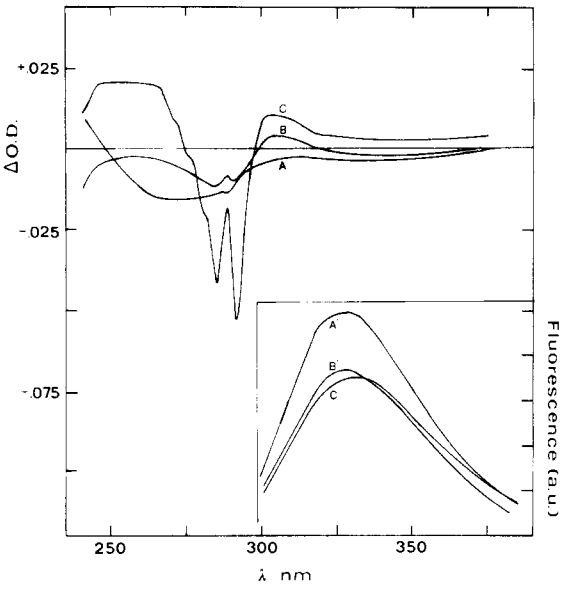


FIGURE 2: Spectroscopic changes induced by hydrogen peroxide on rhodanese. Difference absorption spectra. The base line was made with both sample and reference cuvettes containing 26 μM sulfur-loaded enzyme (ES) in 50 mM phosphate buffer, pH 7.6. (Curve A = E vs. ES) Upon addition of 30 μM cyanide to the sample. To the sulfur-free enzyme a stoichiometric amount of hydrogen peroxide was added. The spectra were taken after 5 min (curve B) and after 50 min (curve C). Fluorescence spectra. Curves A′, B′, and C′ correspond to the samples A, B, and C described above upon 1:10 dilution with buffer. Other experimental details as in Figure 1.

The effect of hydrogen peroxide on sulfur-free enzyme is reported in Figure 2. The addition of 1 equiv of hydrogen peroxide to sulfur-free enzyme is accompanied by a rapid (5-min) quenching of fluorescence (curve B′) and by a relative

Table I: Fluorescence Quantum Yields and Lifetimes of Rhodanese Derivatives

derivative	relative quantum yield × 100, q^a	lifetimes						
		τ_{av} (ns)^b	%^{c}	τ_1 (ns)^d	$\alpha_1{}^e$	τ_2 (ns)^d	$\alpha_2{}^e$	χ^2
E	100	2.6	100	1.7	0.46	3.4	0.54	1.4
ES	77	2.0	76	1.3	0.54	2.8	0.46	1.6
ESe	60	1.5	59	1.0	0.76	3.1	0.24	1.4
E + iodoacetate	68	2.4	90	1.6	0.72	4.5	0.28	1.3
E + hydrogen peroxide	74	2.4	90	1.5	0.72	4.6	0.28	1.1

^{a}Calculated by integration of corrected fluorescence spectra plotted vs. $1/\lambda$ ($\Delta q/q \approx 0.02$). $^b\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$; $\Delta\tau_{av}/\tau_{av} \approx 0.1$. ^{c}Ratio of τ_{av} of a derivative to τ_{av} of E-derivative × 100. $^d\Delta\tau \approx 0.2$ ns. $^e\Delta\alpha \approx 0.05$.

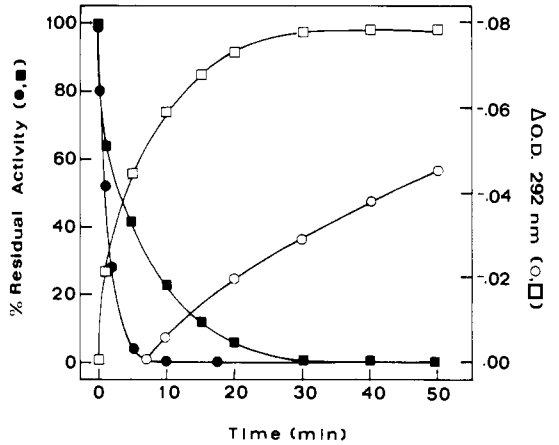


FIGURE 3: Time course of inactivation and absorbance perturbation of rhodanese by hydrogen peroxide or iodoacetate. Sulfur-free enzyme (26 μM) was incubated at 20 °C with an equimolar amount of hydrogen peroxide (circles) or with 50 μM iodoacetate (squares) in 50 mM phosphate buffer, pH 7.6. The reaction was started by addition of each reagent; then at the indicated times, optical readings at 292 nm were measured (empty symbols), and 5-μL aliquots assayed for activity (solid symbols). The amount or free sulfhydryl groups was four before starting the reactions, but only three were found after inactivation of the enzyme. Further incubation ($\simeq$50 min) of the enzyme oxidized by hydrogen peroxide reduced the number of free sulfhydryl groups to two.

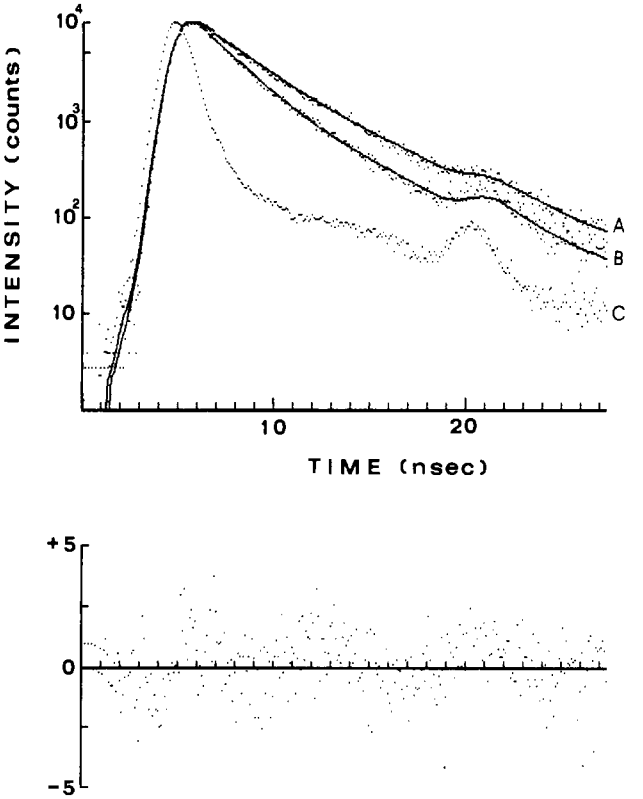


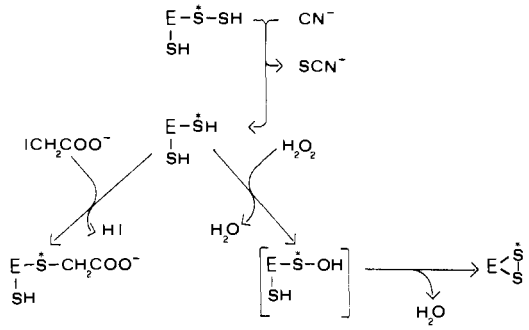
FIGURE 4: Fluorescence decay profiles of rhodanese: (A) sulfur-free enzyme; (B) sulfur-containing enzyme; 10 μM rhodanese in 50 mM phosphate buffer, pH 7.6. Continuous curves refer to the best fit of the data with two exponential decays (see Table I). (C) Lamp profile. Plot of the weighted residuals relative to the sulfur-containing enzyme (B) is reported in the lower panel. All fluorescence decay measurements were made at 20 °C.

increase of absorbance around 260 and above 300 nm (curve B). Upon incubation, the difference absorption spectrum is continuously modified up to 50 min when the spectrum (Figure 2, curve C) is obtained. Minima at 292 and 285 nm are apparently like those observed in Figure 1, but with a lower extinction ($\Delta\epsilon_{292}$ = 1600 M^{-1} cm^{-1}). In the same time the broad absorption around 260 nm continuously increases. The fluorescence emission is not furtherly decreased during this period, but a 5-nm bathochromic shift becomes apparent (Figure 2, curve C'). Both fast and slow phases of the reaction with hydrogen peroxide are faster at higher hydrogen peroxide concentrations. However, it is impossible to derive the relative kinetic parameters since, when hydrogen peroxide is in excess over the enzyme concentration, the solution becomes turbid and the enzyme slowly precipitates. The addition of 10 mM sodium arsenite, just after the completion of the inactivation, restores about 10% of enzyme activity. Thiosulfate (100 mM) restores fully the enzyme activity if added at the end of the rapid phase whilst only 30% after the slow phase is over.

Both reactions of iodoacetate and hydrogen peroxide with sulfur-free enzyme are stoichiometric and accompanied by a loss of enzyme activity (Figure 3) and by a decrease of the number of free sulfhydryl groups. The reaction with iodoacetate brings about in the same time scale the inactivation of the enzyme, the spectral perturbation peaking at 292 and 285 nm, the fluorescence quenching and shift, and the loss of 1 -SH group/mol of enzyme. The reaction with hydrogen peroxide instead causes a rapid quenching of fluorescence and the oxidation of 1 -SH group/mol of enzyme, which parallels the complete loss of enzyme activity. Only after 50 min is there a fully developed absorbance perturbation (Figure 3) and fluorescence shift (Figure 2, curve C') accompanied by the loss of a second sulfhydryl group.

Fluorescence lifetime measurements were performed on the various rhodanese derivatives. Figure 4 shows that the fluorescence decays of sulfur-free and sulfur-containing enzyme are significantly different. The decay profile of each rhodanese derivative can be fitted by using a double-exponential function with two lifetimes: a shorter one τ_1 of 1–1.7 ns and a longer one τ_2 ranging from 2.8 to 4.6 ns (Table I). The ratio of the preexponential factors relative to τ_1 and τ_2 varies between 3 and 1. The weighted residuals relative to the fit of sulfur-containing rhodanese is reported in the lower part of Figure 4 as an example. It shows that the residuals are randomly distributed around zero. Fitting the decays with a single-exponential function is not satisfactory, leading to a χ^2 on the order of 20 and a nonrandom distribution of residuals. On the other hand, fitting the decays with a three-exponential function does not significantly improve the value of χ^2 with respect to the two-exponential function. Table I also shows

Scheme I



that the τ average decrease of the sulfur- and selenium-loaded rhodanese forms is proportional to their quantum yield decrease, while it does not quite account for the fluorescence quenching observed in the carboxymethylated and oxidized derivatives.

Discussion

The intrinsic fluorescence of rhodanese results from eight tryptophans present in the single polypeptide chain (M_r 33000; 293 residues). The maximum of emission (328 nm) indicates that the fluorescent tryptophans could be assigned to class 1 according to Burstein et al. (1973). This class is formed by tryptophyl side chains deeply buried in the interior of a protein, without any interaction with the bulk solvent. The X-ray structure indeed shows that at least seven out of the eight tryptophans are in a hydrophobic environment. Some of them form hydrogen bonds with neighbor amino acid residue either with their peptidic units (Trp-14 and -133) or through their side chains (Trp-112, -113, -275, and -278); finally, the side chain of Trp-35 is a component of the hydrophobic surface area near the active site (Ploegman et al., 1978b). The fluorescence decay of rhodanese (Table I) can be fitted with a biexponential function in analogy with observations made in other multitryptophan proteins (Beechem & Brand, 1985). This finding cannot be taken as indicative of a physical state, i.e., the presence of two classes of tryptophans, due to the presence of several tryptophans in rhodanese. Nevertheless, the fluorescence spectroscopy has proven particularly useful in the study of rhodanese since it can monitor nearly every modification of the active site cysteine. In fact, the derivation of this cysteine is accompanied by spectroscopic perturbations in the UV region. These perturbations may affect both the absorption and the fluorescence of the protein (Figures 1 and 2). In particular, the formation of a persulfide or of a sulfoselenide group in the active site quenches the fluorescence of the protein without affecting its shape. This quencing was attributed to local perturbation by Davidson and Westley (1965) or to a long-range energy transfer by Finazzi-Agrò et al. (1972) and by Guido and Horowitz (1975). This paper shows that the τ_{av} of the two rhodanese derivatives is decreased proportionally to the decrease in the quantum yield (Table I). Furthermore, both the long and short lifetimes are affected in the same way. Therefore, the quenching mechanism should be an energy transfer involving all the fluorescent tryptophans, without major rearrangements of the protein folding. In fact, the difference spectrum of unloaded vs. S-loaded enzyme only shows the disappearance of the persulfide bands at 335 and 270 nm (Figure 1). Analogous results were obtained with the Se-loaded enzyme, which shows difference absorption bands at 375 (very broad), 280, and 260 nm (data not shown). The enzyme can undergo several cycles of loading and unloading of sulfur without significant decrease of enzyme activity (Cannella et al., 1974). Instead, the reaction of sulfur-free enzyme with iodoacetate or hydrogen peroxide leads to a significant perturbation of the UV spectrum and to inactivation of the enzyme due to the chemical modification of the active site cysteine. Iodoacetate brings about carboxymethylation of the sulfhydryl group, which becomes unavailable for reaction with thiosulfate, with the same time course of the spectral perturbation. Hydrogen peroxide oxidizes the enzyme by forming an intramolecular disulfide bridge between Cys-247 and a neighbor cysteine residue as already reported by Weng et al. (1978) (see Scheme I). In fact, in our experiments two sulfhydryl groups are lost after the reaction with hydrogen peroxide, and these -SH groups can be regenerated by incubation with 0.1 M thiosulfate (Cannella & Berni, 1983). The ability of thiosulfate in restoring rhodanese activity has been previously shown by Wang and Volini (1968) for inactivation by nitroaryl compounds. The fluorescence quenching and the disappearance of the first -SH group induced by hydrogen peroxide preceed in time the appearance of the perturbation peaking at 292 nm (Figure 3), the shift of fluorescence emission, and the loss of a second -SH group. During this time interval an absorption at 260 nm is formed. It is tempting to speculate that this absorption is due to the formation of a sulfenyl intermediate (E-S-OH). The formation of a sulfenyl group stabilized by the protein environment upon oxidation under mild conditions of the catalytically active -SH groups of glyceraldehyde-3-phosphate dehydrogenase and papain has been suggested [for review, see Torchinsky (1981)]. The modest reactivation of hydrogen peroxide treated rhodanese by arsenite might support the formation of an intermediate sulfenyl group. This sulfenyl intermediate then reacts with a second -SH group, forming a disulfide bond (Scheme I) that seems to require a conformational change of the protein, giving rise to further absorbance changes (Figure 2, curve C).

Both carboxymethylated and oxidized rhodanese have a quenched and red-shifted fluorescence. In this case, the lifetimes are not decreased proportionally to the fluorescence yield. This indicates that the quenching mechanism is not operating during the excited state of tryptophans. Ground-state interactions of some tryptophans in these two rhodanese derivatives can be inferred also on the basis of the difference absorption spectrum. The difference spectrum is qualitatively but not quantitatively identical in the two cases and is indicative of the transfer of tryptophyl side chains from a hydrophobic to a hydrophilic environment (Andrews & Forster, 1972) as a consequence of the perturbation of the active site. A change of the environment of some tryptophans is also in keeping with the fluorescence bathochromic shift. It is rather difficult to attribute these changes to individual residues due to the complexity of the system, but they seem to point to conformational changes that might account for at least some of the reported discrepancies between crystal and solution conformation of rhodanese.

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Registry No. L-Cys, 52-90-4; S, 7704-34-9; Se, 7782-49-2; ICH_2CO_2H, 64-69-7; H_2O_2, 7722-84-1; selenosulfate, 1343-97-1; rhodanese, 9026-04-4.

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Isotope Exchange at Equilibrium Studies with Rat Muscle Adenylosuccinate Synthetase†

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ABSTRACT: The kinetic mechanism of rat muscle adenylosuccinate synthetase was studied by determining the rates of isotope exchange at equilibrium. A random sequential binding mechanism was indicated for both the forward and reverse reactions. Aspartate, adenylosuccinate, GDP, and P_i were determined to bind in rapid equilibrium. GTP exchanges with both GDP and P_i at the same rate, which is similar to the exchange rate of IMP with adenylosuccinate. Aspartate exchanges with adenylosuccinate at a higher rate than does IMP over the range of concentrations tested. The slower IMP and GTP exchange rates suggest a forward binding mechanism containing a preferred path in which the quaternary complex is most often formed by aspartate binding to the E–GTP–IMP complex. This preferred path is consistent with an interaction between IMP and GTP in the absence of aspartate as determined by isotope scrambling experiments [Bass, M. B., Fromm, H. J., & Rudolph, F. B. (1984) *J. Biol. Chem. 259*, 12330–12333]. However, the products of such an interaction are tightly bound to the enzyme as no partial exchange reactions between adenylosuccinate and aspartate in the presence or absence of P_i were detected.

Adenylosuccinate synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4] catalyzes the formation of adenylosuccinate from aspartate and IMP while converting GTP to GDP and inorganic phosphate (P_i):[1]

$$\text{GTP} + \text{aspartate} + \text{IMP} \rightleftharpoons \text{adenylosuccinate} + \text{GDP} + P_i \quad (1)$$

Initial rate kinetic studies (which include product inhibition and competitive inhibitor studies of adenylosuccinate synthetase from a variety of sources) are consistent with a random Ter Ter sequential mechanism but do not distinguish among proposed reaction mechanisms (Van der Weyden &

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[1] Abbreviations: P_i, inorganic phosphate; IMP, inosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography; PEI–cellulose TLC, poly(ethylenimine)–cellulose thin-layer chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate. In this paper, "substrates" refers to GTP, IMP, and aspartate; "products" refers to adenylosuccinate, GDP, and P_i; and "reactants" refers to all the reacting components (substrates and products). Since every reactant concentration is varied simultaneously, solutions are conveniently identified as a factor (f) by which the reference concentrations are multiplied.

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