Binding of Metal Cyanide Complexes to Bovine Liver Rhodanese in the Crystalline State†

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ABSTRACT: Bovine liver rhodanese, which catalyzes the transfer of sulfur atoms between a variety of sulfur donor and sulfur acceptor substrates, is inhibited by metal cyanide complexes [Volini, M., Van Sweringen, B., & Chen, F.-Sh. (1978) *Arch. Biochem. Biophys. 191*, 205–215]. Crystallographic studies are described which reveal the binding mode of four different metal cyanides to bovine liver rhodanese: $Na[Au(CN_2]$, $K_2[Pt(CN)_4]$, $K_2[Ni(CN)_4]$, and $K_2[Zn(CN)_4]$. It appears that these complexes bind at one common site at the entrance of the active site pocket, interacting with the positively charged side chains of Arg-186 and Lys-249. This observation explains the inhibition of rhodanese by this class of compounds. For the platinum and nickel cyanide complexes virtually no other binding sites are observed. The gold complex binds, however, to three additional cysteine residues, thereby also displacing the extra sulfur atom which was bound to the essential Cys-247 in the sulfur–rhodanese complex. The zinc complex binds to completely different additional sites and forms complexes with the side chains of Asp-101 and His-203. Possible reasons for these different binding modes are discussed in terms of the preference for "hard" and "soft" ligands of these four metal ions.

Rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) from beef liver mitochondria catalyzes in vitro the transfer of sulfane sulfur from thiosulfate to cyanide according to the following two-step mechanism:

$$S_2O_3^{2-} + \text{enzyme} \rightleftarrows \text{enzyme–S} + SO_3^{2-}$$

$$\text{enzyme–S} + CN^- \rightleftarrows \text{enzyme} + SCN^-$$

where enzyme–S is "sulfur–rhodanese" which carries an additional sulfur atom bound to the essential Cys-247. In the absence of cyanide, or other sulfur acceptors, sulfur–rhodanese is an exceptionally stable intermediate.

The biochemical and structural properties have been the subject of several reviews (Sörbo, 1975; Westley, 1977, 1981). The three-dimensional structure determined by X-ray diffraction at 2.5-Å resolution has been described by Ploegman et al. (1978a,b). The amino acid sequence has been determined by Heinrikson and co-workers (Russell et al., 1978).

The molecule consists of a single polypeptide chain containing 293 residues, which is folded into two distinct domains. These domains are very similar in three-dimensional structure which is surprising in view of their large dissimilarity in sequence (Ploegman et al., 1978a,b). The active site is situated close to a cleft in between the two domains, although virtually all side chains essential for the catalysis of the reaction mentioned above are provided by the second domain only (Ploegman et al., 1979).

Biochemical studies aimed at identifying the parts of the molecule required for catalysis had implicated the following groups (Westley, 1977): (i) a sulfhydryl group, (ii) a cationic site formed by either a metal ion or an arginine or lysine residue, and (iii) a strongly hydrophobic region. Suggestions that active rhodanese contained zinc (Volini et al., 1967) could not be confirmed (Bryant & Rajender, 1971; Cannella et al., 1972). Moreover, the crystals used in the X-ray work contained only trace amounts of zinc (Smit et al., 1974). Neither did the final electron density map provide any support for a possible role of Ca^{2+} or Mg^{2+} (Volini et al., 1978) as there was no nonprotein density which could be attributed to a metal ion. In the crystal structure the catalytically important parts mentioned above could readily be identified as (i) Cys-247, (ii) the side chains of Arg-186 and Lys-249, and (iii) the cluster formed by the side chains of Trp-35, Phe-106, Tyr-107, Trp-212, and Val-251 (Ploegman et al., 1979), so there is no need to suppose a divalent metal ion as a cationic site.

Kinetic studies on bovine liver rhodanese have shown that metal cyanide complexes bind quite tightly to the enzyme, thereby acting as effective inhibitors (Volini et al., 1978). In actual fact, $Au(CN)_2^-$ and $Pt(CN)_4^{2-}$ had served as heavy atom derivatives in the determination of the X-ray phases. In particular $Au(CN)_2^-$ had shown an intriguing binding pattern of three sites of high, roughly equal occupancy and one site of rather low occupancy, with this latter site almost coinciding with the main $Pt(CN)_4^{2-}$ binding site.

In order to obtain detailed three-dimensional information on the mechanism of inhibition by metal cyanides, it was decided to use the powerful difference Fourier technique [see, e.g., Blundell & Johnson (1976, Chapter 14)]. The experimental basis of this study was broadened by collecting additional diffraction data for a $Ni(CN)_4^{2-}$ derivative (Ploegman, 1977) as well as for a $Zn(CN)_4^{2-}$ derivative. For these latter two derivatives the sulfur–rhodanese in the native crystals was converted into the active sulfur-free form by treatment with KCN prior to the actual soaking in the nickel and zinc cyanide solutions. The gold and platinum derivatives had been made with crystals which contained sulfur–rhodanese.

It appears that all four metal cyanides bind to rhodanese in a characteristic manner at the active site entrance, thereby explaining the mode of inhibition by these compounds. Additional binding sites occur for the gold and zinc complexes which can be explained by the different affinities of these metals for different types of ligands.

Experimental Procedures

Rhodanese was isolated and purified essentially as described by Horowitz & DeToma (1970). Crystals were grown as described by Drenth & Smit (1971) from solutions of 2 M $(NH_4)_2SO_4$ containing 1 mM $Na_2S_2O_3$. All chemicals were reagent grade, and they were used without further purification.

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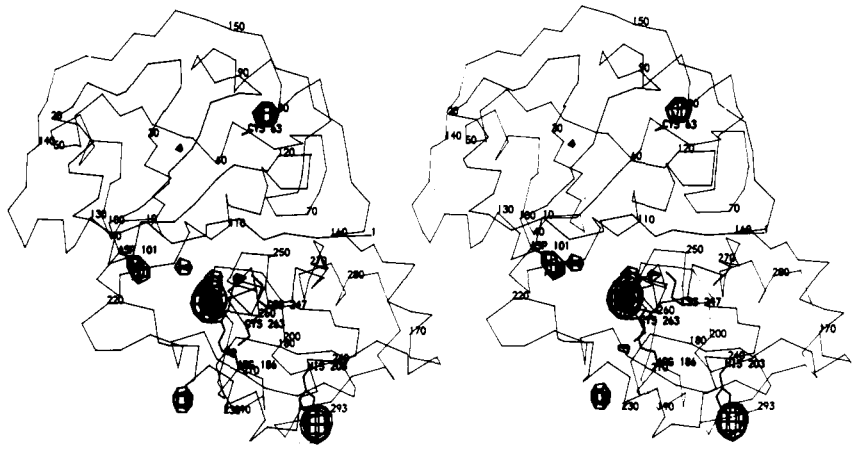

FIGURE 1: Stereo picture showing the positions of the peaks in the $Au(CN)_2^-$, $Ni(CN)_4^{2-}$, $Pt(CN)_4^{2-}$, and $Zn(CN)_4^{2-}$ difference electron density maps together with the C^α chain of rhodanese. Side chains interacting with the bound complexes are shown in bold lines. Cys-247 is drawn as a persulfide and labeled CSS. For the sake of clarity only one positive contour line is shown.

Table I: Rhodanese-Metal Cyanide Complexes: Soaking Conditions[a]

complex	solution and time
$Au(CN)_2^-$	5 mM $Na[Au(CN)_2]$, 6 days
$Pt(CN)_4^{2-}$	10 mM $K_2[Pt(CN)_4]$, 5 days
$Ni(CN)_4^{2-}$	50 mM KCN, 2 × 30 min
	2.0 M $(NH_4)_2SO_4$, 2 × 30 min
	10 mM $K_2[Ni(CN)_4]$, 5 weeks
$Zn(CN)_4^{2-}$	50 mM KCN, 2 × 30 min
	2.0 M $(NH_4)_2SO_4$, 2 × 30 min
	10 mM $K_2[Zn(CN)_4]$, 12 weeks

[a] All solutions contained 2.0 M $(NH_4)_2SO_4$, pH 7.1.

The soaking conditions for obtaining the derivatives are listed in Table I. The widely differing soaking periods were established by taking precession photographs of the centric *h0l* lattice plane until significant differences with respect to the native crystals could be seen.

All diffraction data were collected on a ENRAF-Nonius CAD4-F diffractometer using Ni-filtered Cu Kα radiation from a sealed-off Philips fine-focus tube. Cell parameters calculated from the diffractometer settings were always within 0.3% from those of the native structure. Crystallographic data are summarized in Table II.

The integrated intensities were corrected by Lorentz and polarization factors, as well as for absorption and intensity falloff. The derivative data sets were brought to the same (approximately absolute) scale as the native data set by using a common set of scaling reflections.

Difference electron density maps were calculated on an 1 × 1 × 1 Å grid with the phases calculated from the refined structure at 2.1-Å resolution (L. J. Lijk, G. H. J. van Nes, and W. G. J. Hol, unpublished results). Since errors in the calculated phases are considerable at low resolution, the reflections with $d_{hkl} > 10$ Å were not taken into account. In the difference maps contours were traced at levels of 3 and 6 times the root mean square value calculated over all grid points. The contoured maps were then visualized on an Evans & Sutherland Picture System 2 by using the GUIDE set of programs (Brandenburg et al., 1981).

Results and Discussion

Figure 1 is a composite stereo picture of these four difference maps superimposed on the C^α chain of rhodanese. The side chains in contact with the bound complexes are also shown in thick lines. They all have one site in common: the guanidino group of Arg-186, where in the native structure a sulfate ion is bound as is unequivocally shown in selenate–sulfate exchange studies (L. J. Lijk, C. A. Torfs, K. H. Kalk, M. C. H. De Maeyer, and W. G. J. Hol, unpublished results). The exact positions of the different complexes vary slightly along a straight line through the N^ϵ and C^ζ atoms of this arginine. This is virtually the only binding site for the nickel and platinum complexes. In the case of gold and zinc cyanide, the difference electron density maps also showed peaks at totally different positions on the rhodanese molecule. As will be shown below, at least one of the cyanide ligands seems to have been displaced here by atoms of protein side chains.

Although the thermodynamic data in the literature on the stability of various metal ligand complexes (e.g., Christensen & Izatt, 1970) are of somewhat limited value for the different conditions employed in this study, some trends can be indicated. Such arguments are based on the distinction between "hard" and "soft" ligands: the former are electronegative and form electrostatic interactions (e.g., F^-, H_2O, $R-COO^-$), and the latter are polarizable and may form covalent bonds (e.g., Br^-, R–SH, R–S–S–R′, R_2–NH). Metals are then classified according to their preference for combining with either hard ligands (class a metals) or soft ones (class b metals). From the extensive overview of Blundell & Johnson (1976, Chapter 8), it appears that Pt and Au would prefer soft ligands such as cyanide and sulfur and that Ni and Zn do not have any clear

Table II: Rhodanese-Metal Cyanide Complexes: Crystallographic Data[a]

complex	no. of crystals	$\bar{a}$ (Å)	$\bar{b}$ (Å)	$\bar{c}$ (Å)	β (deg)	minimal Bragg spacing (Å)	no. of independent F_{hkl} values
$Au(CN)_2^-$	11	155.70	49.12	42.13	98.54	3.2	4094
$Pt(CN)_4^{2-}$	9	155.90	49.00	42.19	98.52	3.9	2963
$Ni(CN)_4^{2-}$	6	156.04	49.05	42.13	98.71	2.5	9443
$Zn(CN)_4^{2-}$	5	155.87	49.10	42.14	98.57	4.5	2043

[a] Space group C_2. Cell dimensions of native rhodanese: $a = 156.0$ Å, $b = 49.0$ Å, and $c = 42.2$ Å; $\beta = 98.6°$.

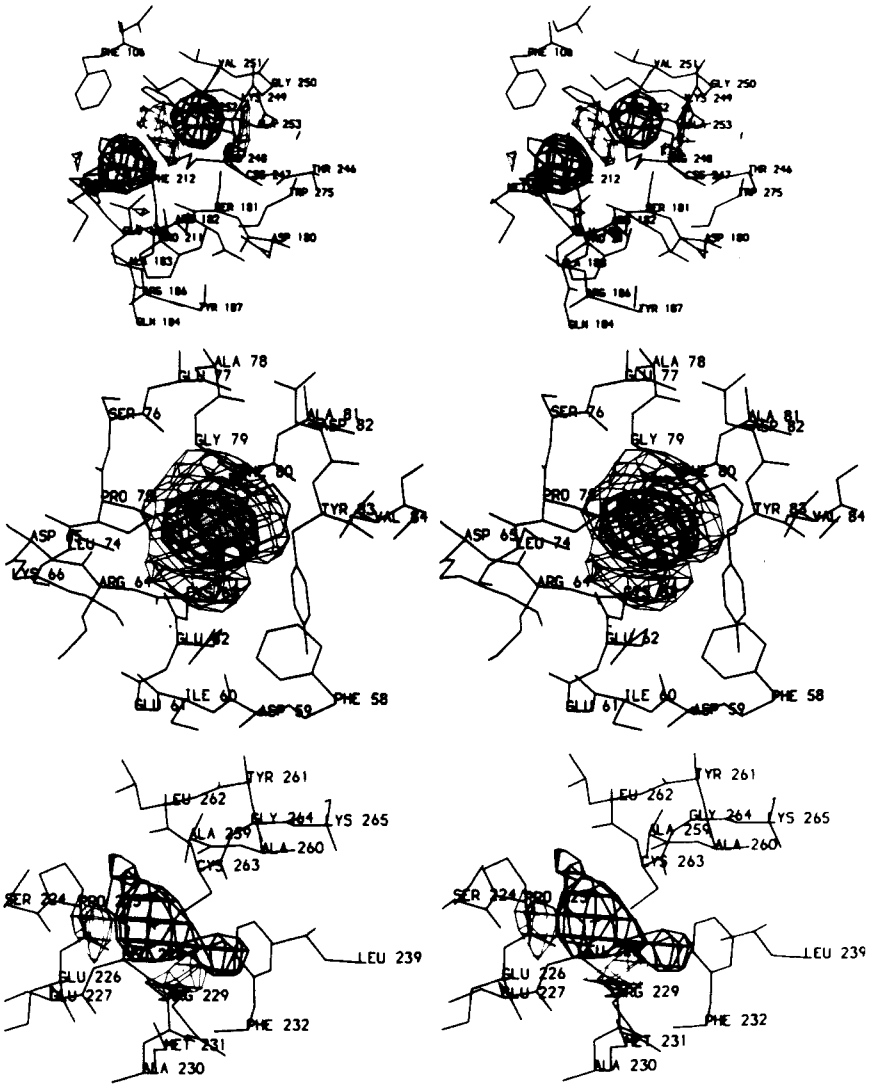

FIGURE 2: Binding sites of gold complexes. (Top) $Au(CN)_2^-$ at about 3.8 Å from Arg-186 and Au–S complex at Cys-247. (Middle) Au–S complex at Cys-63. The negative difference density (thin contours) is the combined result of series termination of the rather limited Fourier summation and the large number of electrons in gold. (Bottom) Au–S complex at Cys-263. In this and all other figures, thin lines indicate negative contour levels and thick lines, positive ones.

preference and may also form complexes with hard ligands such as ammonia or carboxylates.

Binding of Gold Cyanide to Sulfur–Rhodanese. The soaking of sulfur–rhodanese in solutions of gold cyanide yielded four binding sites (Figure 2). The lowest of the four peaks in the difference electron density map is the one near Arg-186. Its relative weakness is probably the result of the fact that it accounts for the difference between the $Au(CN)_2^-$ ion and a bound SO_4^{2-} ion in the native structure. As might be expected for a linear complex (Rosenzweig & Cromer, 1959), the difference density is slighlty ellipsoidal. The three other sites all involve binding to the thiol group of a cysteine residue (Cys-63, Cys-247, and Cys-263). Only the completely internal Cys-254 apparently could not be reached.

It is striking that also Cys-247 is affected, since the crystals had not been treated with KCN prior to soaking with $Au(CN)_2^-$. Nonetheless, the persulfide sulfur present in the native structure is replaced by gold. In view of the soft character of Au^+ (Puddephatt, 1978; Misono et al., 1967) and hence the tendency to form strong bonds with sulfur-containing ligands, a possible explanation of the binding of $Au(CN)_2^-$ to Cys-247 is that cyanide ions displaced from the complex by thiol groups of the accessible residues Cys-63 and Cys-263 react with the persulfide, converting Cys-247 into a "normal" cysteine, which in turn could react with excess $Au(CN)_2^-$.

There can be little doubt that at the three cysteine sites the gold is bound almost covalently to the thiol sulfur: in all cases lies the center of the peak between 2.0 and 3.3 Å from the S^γ at an angle with respect to the C^β–S^γ bond of about 98° such as expected for an atom bound to sulfur.

Binding of Nickel Cyanide to Rhodanese. Soaking crystals of rhodanese in solutions of nickel cyanide yielded one major and one minor site. The major site is the common position at the entrance of the active site pocket. Because of the high resolution of the diffraction data of the $Ni(CN)_4^{2-}$ derivative, both the peaks and the holes are better defined than in any of the other difference electron density maps discussed here (Figure 3). From the high spherical peak near Arg-186 two protrusions extend: one toward Cys-247 and another toward Lys-249. Most probably they represent some solvent particles lining up with the cyanide complex. The negative density (in thin lines) shows that the persulfide S^δ atom has been removed by the treatment with KCN. Another, kidney-shaped, positive density is observed near S^γ-247, which almost certainly stems from oxidation of the thiol group to a sulfinic acid. This $-SO_2$ group can form hydrogen bonds with the amide groups of Gly-250 and Ala-253 and with the hydroxyl groups of Ser-181, Thr-252, and Ser-274. A low spherical peak between the side

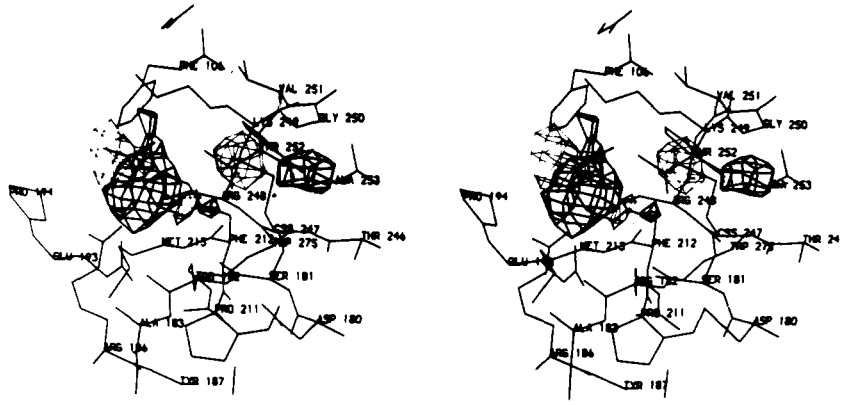

FIGURE 3: Binding of the $Ni(CN)_4^{2-}$ complex at about 4.2 Å from Arg-186. The negative densities around the main positive peak reflect changes in the solvent structure.

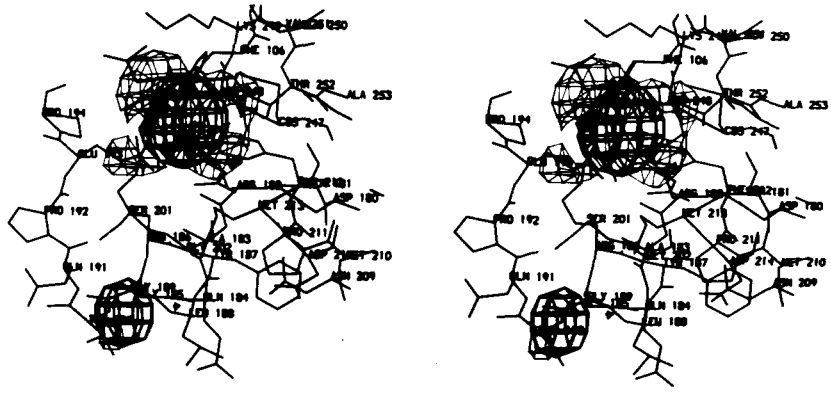

FIGURE 4: Binding of the $Pt(CN)_4^{2-}$ complex at about 4.2 Å from Arg-186. The persulfide sulfur of Cys-247 is behind the negative difference density and not sticking into it.

chains of Gln-184 and Gln-191, which is also seen in the platinum difference Fourier, may be due to a change in the mode of binding of some solvent molecules.

Binding of Platinum Cyanide to Sulfur–Rhodanese. Just like nickel cyanide, platinum cyanide binds at one major and one minor site to rhodanese (Figure 4). The major site is the position near Arg-186, common to all four metal cyanides, and the minor position coincides with the minor nickel position (not shown in Figure 3). It is obvious that the platinum compound binds to sulfur–rhodanese as the positive and negative features near S^{γ}-247 in the nickel difference electron density map are absent in the case of platinum.

The platinum compound behaves distinctly different from the gold complex: no complexation with an sulfhydryl group is observed, and the persulfide S^{δ} of Cys-247 is not replaced by platinum. This is consistent with the explanation given for the removal of this sulfur atom by gold cyanide: in the case of platinum sulfur ligands are apparently unable to displace cyanide from the metal complex, and hence no cyanide ions are available which can remove the extra sulfur from Cys-247.

The $Ni(CN)_4^{2-}$ and $Pt(CN)_4^{2-}$ complexes are both planar (Brasseur & de Rassenfosse, 1937; Krogmann, 1969). This geometrical similarity produces an accordingly similar change in solvent structure as indicated by the same minor peak near Gln-184 in both difference electron density maps, which is not seen in the cases of gold and zinc.

Binding of Zinc Cyanide to Rhodanese. Soaking in zinc cyanide solutions yielded three binding sites in crystalline rhodanese. The binding mode of this complex clearly underlines the intermediate position of zinc between class a and class b metals. Although here too, the cyanide complex binds near Arg-186 (Figure 5, top), there are two other binding sites which clearly do not represent the cyanide complex: one at Asp-101 and one at His-203. In the latter case the imidazole $N^{\epsilon 2}$ atom is about 1.8 Å from the center of the difference density peak (Figure 5, bottom). At Asp-101 the two oxygen atoms of the carboxyl group are 1.3 and 3.1 Å from the center of the peak (Figure 5a). Assuming a slight rotation around the C^{α}–C^{β} bond, an equal distance of about 2.1 Å could be obtained. In both cases, it is impossible to tell from the difference electron density map what the other, nonprotein, ligands to the zinc atom are: either CN^-, NH_3, or H_2O.

In trying to understand why no other complexes between zinc and rhodanese are formed, it is worthwhile to look at alternative places. Among the remaining seven histidine residues only His-120 could possibly provide an accessible imidazole. Its environment, however, is quite different from that of His-203. Whereas the former is fully exposed to the solvent, the latter is tucked away between loops formed by residues 201–209 and 286–290. Although none of these residues bind directly to the complex, they may have helped in its formation via solvent molecules.

Among the many carboxyl groups Asp-101 clearly is a special case. Its side chain lies in the N-terminus area of both helices D and E, close to the intersection of the helix axes. Hence, the helix dipoles (Hol et al., 1978) may have helped to stabilize a negatively charged complex involving the carboxyl group. The fully exposed Asp-102 (cf. Figure 1), which is only 4 Å away from Asp-101, does not show any binding of zinc complexes at all. This is in agreement with the fact that the dielectric screening by water increases rapidly going from the surface of a protein molecule out into solution.

As in the case of nickel cyanide, the extra sulfur atom in the active site has been removed by the treatment with KCN, and the remaining S^{γ}-247 has been oxidized. It is interesting to note that this oxidation is considerably less pronounced than

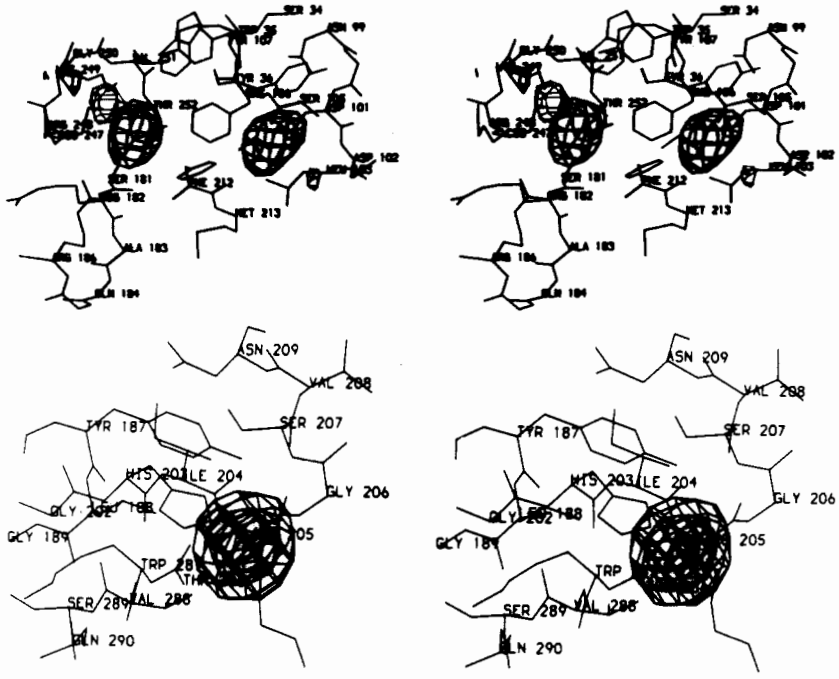

FIGURE 5: Binding of zinc complexes to rhodanese. (Top) $Zn(CN)_4^{2-}$ at about 4.6 Å from Arg-186 and zinc–carboxyl complex near Asp-101. (Bottom) Zinc–imidazole complex near His-203.

in the case of nickel, although the soaking period had been twice as long. This may be due to the different geometries of the complexes. The peak of the tetrahedral $Zn(CN)_4^{2-}$ complex (Sequeira & Chidambaram, 1966) is shifted about 0.5 Å away from Arg-186 with respect to the position of the planar nickel and platinum complexes, probably resulting in a more effective shielding of Cys-247.

Conclusion

The common mode of binding of metal cyanides to rhodanese and to sulfur–rhodanese is near Arg-186 at the entrance to the active site pocket. This observation provides a direct explanation for the kinetically observed inhibitory effect of this class of compounds (Volini et al., 1978).

Arg-186 in the only internal arginine residue where the guanidino group does not join with other groups in salt or hydrogen bridges, and so it is readily understood that it is the cornerstone of the cationic site required for the catalytic reaction. Another reason for the great preference for negatively charged groups to bind close to Arg-186 may be the presence of the ζ-amino group of Lys-249 at a distance of about 5 Å from the common metal cyanide binding site. Although the precise contribution to the binding energy of anions by this lysine residue is hard to estimate, it is reasonable to assume that this is the decisive factor.

Of the four metals investigated in the present study, two behave in a very similar way: platinum and nickel. This is in spite of the fact that the former falls in the class b metals and the latter in an intermediate class between class a and class b. Nickel, in this case, displays a class b preference for the soft ligand cyanide. Zinc also falls in this intermediate class but evidently behaves accordingly by complexing both with cyanide and with the harder imidazole and carboxylate ligands. The gold complexes show the class b nature of the metal by complexing only with the soft ligands cyanide and sulfur.

Why does platinum cyanide bind in such a different way compared to gold cyanide? Both metals involved prefer soft ligands, as mentioned above, and the stability constants of these complexes in aqueous solution are quite similar (Martell & Sillen, 1964; Puddephatt, 1978)—both in the order of 10^{40}. A possible explanation may be the small rate of dissociation of the platinum cyanide complex (Basolo & Pearson, 1967, p 382). Another factor may be the requirement of Pt^{2+} to be coordinated by four ligands, whereas Au^+ can form stable complexes with just two ligands. The somewhat buried nature of the three cysteine residues of rhodanese which form complexes with Au^+ may prohibit complex formation with Pt^{2+} and three additional ligands.

Both intermediate types of metals used in this study, Zn^{2+} and Ni^{2+}, also show a distinctly different behavior. This can be explained neither by a slow rate of dissociation of the nickel complex, as the cyanide occurs readily (Basolo & Pearson, 1967, p 417), nor by a different number of ligands preferred by these two ions. In addition, the stability constants for complexes with hydroxyl ions, acetate, and ammonia are very similar for these two metals (Misono et al., 1967), which implies that the affinity for the carboxyl and imidazole groups of rhodanese would also be approximately the same for Ni^{2+} and Zn^{2+}. Other factors must therefore cause the differences in binding to rhodanese. Ligand field stabilization of $Ni(CN)_4^{2-}$ species is considerable but does not occur with the d^{10} metal ion Zn^{2+}, and this leads to a distinct difference in stability of the cyanide complexes. The association constant is about 10^{30} for the nickel complex (Christensen et al., 1963) but only 10^{20} for the zinc compounds (Izatt et al., 1965). Although these values have been determined in aqueous solutions and may not be fully applicable to the high ionic strength conditions of the present investigation, it seems reasonable to assume that different stabilities of the nickel and zinc complexes cause the different binding modes to rhodanese.

It appears that in 2 M ammonium sulfate the binding sites of metal cyanides are hard to predict. The behavior of zinc cyanide suggests that this compound, despite the relatively low atomic weight of zinc, may be useful in preparing heavy atom derivatives in certain cases.

As our crystallographic studies are related to the kinetic studies of Volini et al. (1978), it may be pointed out that good agreement does exist as to the inhibition by the zinc and nickel cyanide ions. As to the presence of Mg^{2+} and Ca^{2+} in rhodanese, there is no crystallographic evidence that these ions

are tightly bound in the active site. Even though the solvent surrounding the enzyme molecules contains 2 M ammonium sulfate, this should not have prevented the observation of a tightly bound Ca^{2+} ion as at least one case is known where such an ion was observed in the presence of a high ammonium sulfate concentration (Kretsinger & Nockolds, 1973). It is somewhat remarkable in the studies of Volini et al. that 1 g-atom of metal ion is bound per 18 500 daltons, whereas the molecular weight of bovine liver rhodanese has been established by a number of widely varying techniques to be 33 000 (Bergsma et al., 1975; Russell et al., 1975; Trumpower et al., 1974). This would mean that about 2 g-atoms of Ca^{2+} or Mg^{2+} would be bound by rhodanese which is in contrast with the fact that rhodanese contains only a single active site (Ploegman et al., 1979). Nevertheless, the crystallographic studies do not directly rule out the possibility that Ca^{2+}, Mg^{2+}, or Zn^{2+} may assist the enzyme by a transient binding to a catalytic intermediate. Such a metal-activated catalysis, however, seems to be somewhat superfluous as the two positive charges of Arg-186 and Lys-249 at the entrance of the active site pocket seem to be well placed to assist in the catalysis of rhodanese by binding and polarizing the negatively charged substrates of this enzyme (Ploegman et al., 1979; Hol et al., 1983).

Acknowledgments

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Registry No. Rhodanese, 9026-04-4; $Na[Au(CN)_2]$, 15280-09-8; $K_2[Pt(CN)_4]$, 562-76-5; $K_2[Ni(CN)_4]$, 14220-17-8; $K_2[Zn(CN)_4]$, 14244-62-3; Arg, 74-79-3; Lys, 56-87-1; Cys, 52-90-4; Asp, 56-84-8; His, 71-00-1.

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