Cobalt and Ruthenium Replacement for Iron in Adrenal Iron–Sulfur Protein (Adrenodoxin). Preparation and Some Properties†

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ABSTRACT: The Co- and Ru-substituted derivatives of adrenal iron–sulfur protein (adrenodoxin) were prepared from its apoprotein in the presence of urea, dithiothreitol, Na_2S, and metal ions. Both metal-substituted proteins had 2 g-atoms each of metal and labile sulfur per mole of protein. The Co derivative had optical absorption maxima at 257, 264, 470, and 1430 nm with shoulders at 275, 280, 300, and 380 nm. The molar extinction coefficient per Co atom was 2.200 M^{-1} cm^{-1} at 470 nm. The Ru derivative had a broad maximum at 500 nm with a molar extinction coefficient of approximately 100 M^{-1} cm^{-1} per Ru atom. The visible chromophore of the Co-substituted protein was stable in the pH region from 7.5 to 9.5, which resembled that of the native protein. The titration of the Co- and Ru-substituted proteins with mericurials revealed that the saturation levels are 8.6 and 8.4 mol of mercurial/mol of protein. The values agree with that of the native protein within experimental errors. The tyrosyl residue at position 82 displayed a broad anomalous emission at 335 and 331 nm for the Co- and Ru-substituted proteins, respectively, as well as in the case of the native protein. There was no electron paramagnetic resonance signal of the Co derivative in a wide magnetic field at 77°K. Additionally, the Co and Ru derivatives had no enzymatic activity toward NADPH–cytochrome *c* reduction in the presence of adrenal diaphorase (adrenodoxin reductase). There was no indication that Mn, Ni, Cu, and Os are incorporated into the apoprotein in the presence of urea. Incorporation of Fe into the protein was examined in the presence of Co or Ru. In a system containing both Fe and Ru, Fe was exclusively incorporated into the protein. In contrast to this, the reaction products from a system containing both Fe and Co were found to consist of both Fe and Co derivatives at approximately equimolar quantity.

Adrenal iron–sulfur protein (adrenodoxin) serves as an electron carrier in a system which hydroxylates steroids in the presence of NADPH and molecular oxygen. The oxidation–reduction center, which consists of two iron atoms, two labile sulfur atoms, and four cysteine residues, is believed to be a binuclear iron–sulfur cluster, $[Fe_2S_2(SR)_4]$.

We have previously prepared the selenium-substituted derivative of the adrenal protein by replacement of the native labile sulfur atoms, and its properties have been extensively studied (Mukai *et al.*, 1973, 1974; Bowman *et al.*, 1973). Subsequently, we decided to prepare the metal-substituted derivatives of the adrenal protein. Our hopes were (1) to know the specificity of the protein toward metal incorporation, (2) to see whether or not the metal-substituted proteins undergo enzymatic catalysis, and (3) to gain some insight into the chromophore utilizing the vast knowledge concerning cobalt coordination complexes.

Although tremendous amounts of work on iron–sulfur proteins have been carried out in the past, no report of metal substitution in iron–sulfur proteins has been published in the literature. We wish to report both the preparation as well as some of the properties of the Co- and Ru-substituted proteins.

Materials and Methods

Preparation of Co- and Ru-Substituted Proteins from Adrenal Iron–Sulfur Protein. Bovine adrenal iron–sulfur protein was prepared by the method described previously (Kimura, 1968). The ratio of the absorbance at 414 nm to that at 276 nm was 0.86. The apoprotein, which was prepared by a method reported elsewhere (Mukai *et al.*, 1974) had negligible amounts of labile sulfur and iron atoms. The Co- and Ru-substituted proteins were prepared by the following method. A solution (3.0 ml) containing 20 mg of the apoprotein in a 0.5 M Tris buffer (pH 7.8) was placed in a reaction vessel; a 25-fold molar excess of dithiothreitol over the protein was added to the apoprotein solution and urea was added to make a 6 M solution. After 3 hr at 0°, a threefold molar excess of Na_2S and a threefold molar excess of either $CoCl_2$ or $(NH_4)_3RuCl_6$ in 0.5 ml of H_2O were added to the reaction mixture. When the solution of $CoCl_2$ was added to the reaction mixture, a yellowish brown color immediately appeared. The reaction mixture was then kept for 30 min at 0°. (In the case of ruthenium, the reaction was continued for 3 hr at 0° with occasional stirring.) At the end of the period, a pale pink color appeared. Then the reaction mixture was placed on a small DEAE-cellulose column (0.7 × 8 cm) and subsequently washed with 100 ml of 0.01 M phosphate buffer (pH 7.8) containing 0.17 M KCl. The metal-substituted protein was eluted with 0.01 M phosphate buffer (pH 7.8) containing 0.50 M KCl. The eluate was further applied onto a Sephadex G-25 column (1.0 × 10 cm) equilibrated with 0.01 M phosphate buffer (pH 7.8) and was eluted with the same buffer (pH 7.8) containing 0.3 M KCl. The selenium derivative was prepared by the same method except H_2SeO_3 was used instead of Na_2S.

Other Materials. Adrenal NADPH-diaphorase (adrenodoxin reductase) was prepared by the method described by Chu and Kimura (1973). Cytochrome *c* (type III), NADPH, and dithiothreitol were obtained from Sigma.

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received July 18, 1974.* This study was supported by a Research Grant from the National Institutes of Health (AM-12713).

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TABLE I: Chemical Analyses of Cobalt, Ruthenium, Sulfur, and Protein in Cobalt- and Ruthenium-Substituted Adrenodoxins.

	Chemical Analysis (mM)						
	Protein	Sulfide	Iron	Co	Ru	S/Metal	Metal/Protein
Native adrenodoxin	0.336	0.630	0.654			0.96	1.95
Co-substituted adrenodoxin	0.280	0.506		0.464		1.09	1.66
Ru-substituted adrenodoxin	0.371	0.650			0.574	1.13	1.55

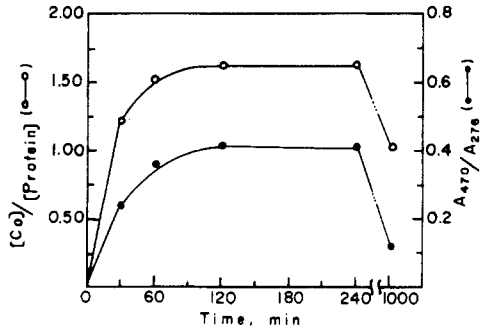

FIGURE 1: Correlation between time of treatment with 6 M urea and incorporation of cobalt with apoadrenodoxin. Apoadrenodoxin (1 mM) was treated with 6 M urea for the indicated time. Then 3 mM $CoCl_3$ and 3 mM Na_2S were added to the solution in the presence of 25 mM dithiothreitol. The amount of incorporation of the metal and the absorbance at 470 nm were measured.

Other reagents were of the best grade available from commercial sources.

Analytical Methods. Iron was determined by the *o*-phenanthroline method (Kimura and Suzuki, 1967). Cobalt was determined by the spectrophotometric methods of Anand *et al.* (1961) and Dewey and Marston (1971) after precipitating the protein with 5% trichloroacetic acid. The determination of ruthenium was carried out by the method of Embry and Ayres (1968). Ruthenium reacted with this reagent at pH 2.2–4.0 to give a red-purple complex having an absorption peak at 510 nm. Labile sulfur was determined by the Methylene Blue method (Kimura and Suzuki, 1967). Protein concentration was determined by the biuret method.

Other Methods. NADPH-cytochrome *c* reductase activity was measured by the method described previously (Chu and Kimura, 1973). Spectrophotometric measurements were carried out by the use of a Hitachi spectrophotometer (Model 124). An automatic recording spectrofluorometer was used for all fluorescence measurements. Electron paramagnetic resonance (epr) spectroscopy was carried out by a Varian spectrometer (Model E-4) at 77°K.

Results

Substitution Attempts with Various Metal Ions. An attempt to substitute the iron atoms of the adrenal iron–sulfur protein with Mn, Co, Ni, Cu, Ru, and Os was made using a system consisting of the apoprotein (1 mM), metal ion (3 mM), and sodium sulfide (3 mM). Metal analysis and spectrophotometric measurement of the resulting compounds revealed that in the presence of 25 mM dithiothreitol and 6 M urea, Co and Ru, as well as iron, were incorporated into the apoprotein at yields of 83 and 78%, respectively, while Mn, Ni, Cu, and Os were not. In the absence of urea, there was no indication of the incorporation into the protein of any of the metal ions tested, except for iron.

It is of interest that Co and Ru are at neighboring positions with Fe in the periodical table. However, it was difficult to correlate incorporation of the metal with Pauling's metal ion radii (Mn^{2+} 0.80 Å; Fe^{3+}, 0.60 Å; Fe^{2+}, 0.74 Å; Co^{2+}, 0.72 Å; Ni^{2+}, 0.69 Å; Cu^{2+}, 0.72 Å; Ru^{4+}, 0.67 Å; and Os^{4+}, 0.88 Å).

Table I shows the chemical analysis of the Co- and Ru-substituted samples in comparison with that of the native iron protein, indicating that these compounds contain both metal and labile sulfur atoms at equimolar ratio. From these results, it is conclude that the Co and Ru derivatives have 2 g-atoms each of metal ion and labile sulfur per mole of protein.

Cobalt Incorporation as a Function of Time of Urea Treatment. Figure 1 shows the correlation between the incorporation of Co into the apoprotein with the time of the treatment with 6 M urea. The amounts of incorporation of the metal increased with the length of treatment up to 2 hr; then the amount of incorporation gradually decreased. Additionally, it was noted that the rate of the Co incorporation is comparable to that of the increase in the absorbance at 470 nm.

Optical Absorption Characteristics. The ultraviolet and visible absorption spectra of the Co-substituted and native adrenal iron–sulfur protein are shown in Figure 2A. The Co derivative had peaks at 257, 264, and 470 nm with shoulders at 275, 280, 300, and 380 nm. The ultraviolet spectrum below 300 nm was very similar to that of the native protein. The molar extinction coefficient was determined as 2200 M^{-1} cm^{-1} per cobalt atom at 470 nm. In fact, the Co complexes with sulfur containing ligands such as 2-mercaptoethanol and 1,3-propanedithiol exhibited absorption maximum near 500 nm with the molar extinction coefficient having an order of magnitude of 10^3.

Since Co(III) and Fe(II) have the d^6 configuration of electrons, it is worthy to compare the Co-substituted protein with the reduced form of adrenal iron–sulfur protein. As reported previously (Kimura, 1968), the reduced protein has an absorption maximum at 550 nm, which is far from the band at 470 nm of the Co derivative. This would suggest that the metal ion is Co(II) rather than Co(III) (see Discussion).

In order to see the d–d transition bands in the near-infrared region, we have carried out the measurements by using a D_2O-replaced sample of the Co-substituted protein (Figure 2B). Over the background absorption of the apoprotein in D_2O, the Co protein exhibited an absorption band at 1430 nm. To check the contamination of H_2O into the sample, the absorption bands of H_2O were detected by adding a small amount of H_2O into the sample. The band at 1430 nm would be assigned a d–d transition band of Co(II) in tetrahedral symmetry. The absorption characteristics are summarized in Table II along with their tentative assignments.

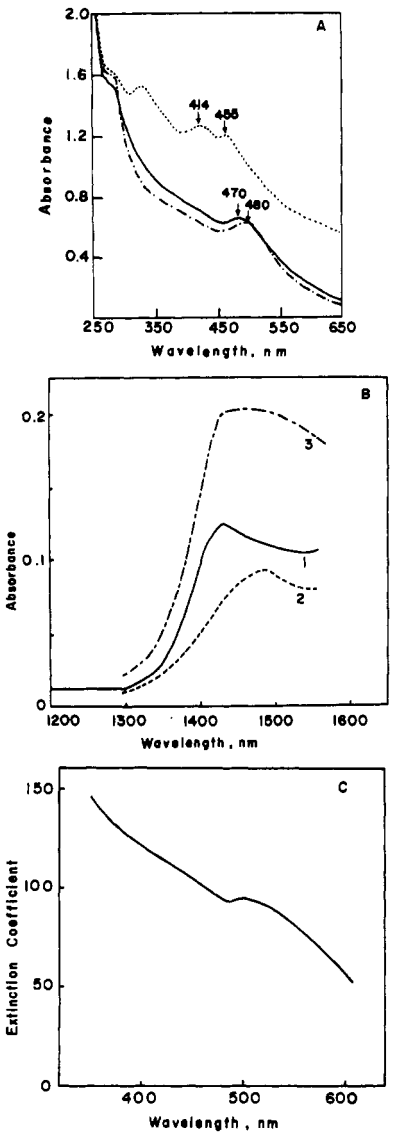

FIGURE 2: (A) Optical absorption spectra of cobalt-substituted adrenodoxin: (—) cobalt-substituted adrenodoxin; (- · -) cobalt- and selenium-substituted adrenodoxin; (- - -) native adrenodoxin. The concentration of the protein was 0.15 mM. The pH of the solution was 7.8. From these data, the molar extinction coefficients of cobalt- and selenium-substituted adrenodoxins at 470 and 480 nm were calculated to be 4400 and 4200 cm^{-1} M^{-1}, respectively, while that of native adrenodoxin was 9800 at 414 nm. (B) Near-infrared absorption spectrum of the cobalt derivative of adrenodoxin: (1) the sample cuvet contained 0.5 mM Co-adrenodoxin in D_2O and the reference cuvet had 0.5 mM apoadrenodoxin in D_2O; (2) the sample cuvet contained a small amount of H_2O in D_2O, and the reference cuvet had pure D_2O; (3) to the sample cuvet of curve 1, a small amount of H_2O was added; the reference cuvet had 0.5 mM apoadrenodoxin in D_2O. (C) Optical absorption spectrum of ruthenium-substituted adrenodoxin. The protein concentration was 1 mM.

The selenium derivative of the Co protein was prepared by using H_2SeO_3 instead of Na_2S. The resulting selenium compound displayed a maximum at 480 nm, which had a shift of 10 nm toward longer wavelengths than the sulfur complex (Figure 2A). This red shift is within reasonable expectation when the atomic number of chalcogens for a ligand increases.

The Ru-substituted protein prepared in the presence of sodium sulfide had a broad absorption maximum at 500 nm (Figure 2C). The molar extinction coefficient was approximately 100 M^{-1} cm^{-1} per ruthenium atom. The ultraviolet

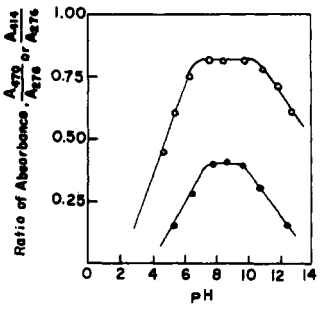

FIGURE 3: pH-stability of cobalt-substituted adrenodoxin (●) and native adrenodoxin (○). The concentrations of cobalt-substituted adrenodoxin and native adrenodoxin were 0.060 and 0.055 mM, respectively. Phosphate and borate buffers were used for the adjustment of pH. The spectra were taken after standing overnight at 0°.

TABLE II: Absorption Characteristics of Co-Substituted Adrenodoxin in Tetrahedral Symmetry of d^7 Configuration.

Absorption Maxima, nm (cm^{-1})	Molar Extinct. Coeff.[d] (M^{-1} cm^{-1})	Tentative Assignment
400 (25,000)[a]	2700	Co–S charge transfer
470 (21,300)	2200	Co–S charge transfer
520 (19,200)[b]		d–d; $^4A_2$–$^4T_1$ (P)
1430 (7000)	~100	d–d; $^4A_2$–$^4T_1$ (F)
2560 (3900)[c]		d–d; $^4A_2$–$^4T_2$

[a] Broad absorption. [b] This band is heavily overlapping with the 470-nm band. A broad absorption around 500 nm would correspond to this transition. [c] This band is predicted theoretically. [d] The values are expressed per cobalt ions present.

spectrum below 300 nm was similar to that of the native protein. Upon the addition of dithionite, both the Co and Ru proteins could not be reduced regardless of the addition of methyl viologen as a mediator.

Stability and pH Effects. The stability of the Co- and Ru-substituted proteins was tested by treating the samples in 0.5 M KCl plus 0.01 M phosphate buffer (pH 7.8) at 37°. The changes in absorbance at the respective maxima were followed. After 3 hr at 37°, the Co- and Ru-substituted proteins decomposed by about 15 and 35%, respectively. When selenium replaced sulfur in the Co derivative, it was more unstable than that of the Co derivative with labile sulfur (25% after 3 hr at 38°).

Figure 3 illustrates the pH effects on the Co-substituted and native proteins. The Co-substituted protein was stable in the pH region from 7.5 to 9.5, and the native protein from 7.0 to 10.5, indicating that both proteins have similar pH profiles. This suggests that both proteins may involve the same ligands in the protein.

Titration with Mercurial Reagents. The Co- and Ru-substituted proteins rapidly react with *p*-hydroxychloromercuribenzoate and *p*-chloromercuriphenylsulfonate, as evidenced by the decrease in absorbance at 470 or 500 nm and the increase in absorbance at 250 nm. As shown in Figure 4, the titration experiments indicate that the saturation levels are 8.6 and 8.4 mol of the mercurial/mol of the Co- and Ru-substituted proteins. Thus, the results strongly suggest that the metal ion is bound to sulfur atoms. Since

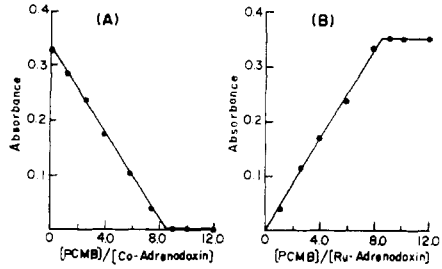

FIGURE 4: Titration of metal-substituted adrenodoxin with *p*-hydroxychloromercuribenzoate. The reaction mixture contained Co-substituted adrenodoxin (0.073 mM) (A) and Ru-substituted adrenodoxin (0.014 mM) (B) in Tris buffer (pH 7.6). To the reaction mixture various amounts of *p*-hydroxychloromercuribenzoate were added and the changes of absorbance at 470 (A) and 250 nm (B) were measured at 20°, immediately after the addition of *p*-hydroxychloromercuribenzoate. The control had the same amount of *p*-hydroxychloromercuribenzoate and Tris buffer.

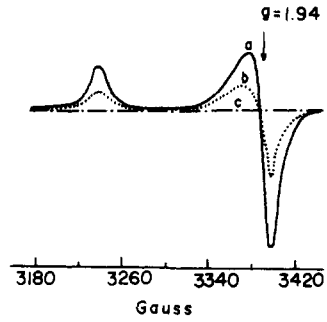

FIGURE 5: Epr spectra of native adrenodoxin and cobalt adrenodoxin: (a) native adrenodoxin; (b) Fe- and Co-substituted adrenodoxin; (c) Co-substituted adrenodoxin. The concentration of the protein samples was 0.5 mM. Conditions of epr spectroscopy: microwave power, 9.8 mW; frequency, 9.285 GHz; modulation amplitude, 5 G; time constant, 1 sec; temperature, 77°K.

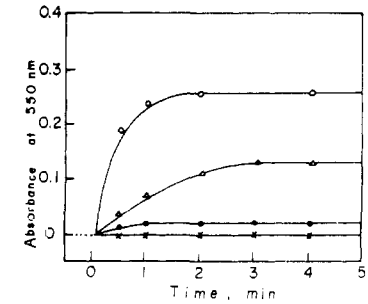

FIGURE 6: NADPH-cytochrome *c* reductase activity of cobalt- (●) and ruthenium- (×) substituted adrenodoxin, cobalt-iron adrenodoxin (△), and native adrenodoxin (○). The concentrations of the components were: metal-substituted adrenodoxin or native adrenodoxin, 1.50×10^{-7} M; adrenodoxin reductase, 0.75×10^{-7} M; cytochrome *c*, 2.85×10^{-5} M; NADPH, 3.80×10^{-5} M; in 3 ml of 0.01 M sodium phosphate buffer (pH 7.6), assay temperature, 25°.

this adrenal protein has five cysteine residues and two labile sulfur atoms per molecule and the mercurial titration of the native protein was 9 mol of mercurial/mol of protein, the Co-S and Ru-S coordination structures appear to be closely similar to that of the native chromophore.

Tyrosine Fluorescence. We have previously observed a broad anomalous emission (331 nm) of the tyrosyl residue at position 82 of the native protein which has no tryptophan and only one tyrosine residue. The apoprotein, free from iron and labile sulfur atoms, exhibited a normal tyrosine emission at 304 nm (Kimura *et al.*, 1972). When excited at 280 nm, the Co- and Ru-substituted proteins displayed a similar anomalous emission with maxima at 335 and 333 nm, respectively. The excitation maxima of the native, Co- and Ru-substituted proteins, and the apoprotein were found to be 284, 286, 284, and 281 nm, respectively. Thus, the Co- and Ru-replaced proteins provide the same anomaly of tyrosine emission as the native protein, suggesting that there is some weak interaction between the tyrosine residue and the metal.

Electron Paramagnetic Resonance. Electron paramagnetic resonance (epr) spectroscopy of the Co-substituted protein was performed at the protein concentration of 1.0 mM at 77°K. There was no detectable signal in a wide magnetic field. This fact is suggestive of the diamagnetic nature of the complexes, although epr signals of high-spin Co(II) are broad and hard to detect (in the tetrahedral field, only the high-spin state is possible for the Co(II) complex). No signal was observed upon the addition of dithionite even in the presence of methyl viologen as a mediator.

Catalytic Activity. The enzymatic reduction of the Co- and Ru-substituted proteins was examined in the reaction mixture containing NADPH, adrenal diaphorase (adrenodoxin reductase), and the metal-substituted protein in an anaerobic cell. The reaction was followed by changes in absorption at 470 or 500 nm. There was no detectable change in the absorbance for a prolonged reaction period. Furthermore, the NADPH–cytochrome *c* reductase activity which is mediated by both adrenal iron–sulfur protein and its reductase was examined by using the Co- and Ru-substituted proteins. Again, there was no detectable reduction with these derivatives. Therefore, it was concluded that the Co- and Ru-substituted proteins have no enzymatic activity toward the electron transfer reaction of adrenal steroid hydroxylases. This is in sharp contrast with the selenium-substituted iron protein which has a comparable activity toward the reduction of cytochrome *c* with the native protein (Mukai *et al.*, 1974).

Competition of Incorporation between Iron and Cobalt or Ruthenium. Incorporation of Fe into the protein was examined simultaneously in the presence of Co or Ru. The reaction mixture contained the apoprotein which was pretreated in 6 M urea for 120 min and a threefold excess (relative to protein) of an equimolar mixture of $FeCl_3$ and $CoCl_2$ or $(NH_4)_3RuCl_6$. After standing for 30 min, the product was separated as described in Methods and Materials.

In a system containing both Fe and Ru, Fe was exclusively incorporated into the protein. In contrast to this, the reaction products from a system containing Fe and Co were found to consist of both Fe and Co derivatives at approximately equimolar quantity. The solution of the reaction products gave an optical absorption spectrum which can be simulated as a composite, containing equimolar amounts of the Fe and Co proteins. As shown in Figure 5, the epr spectrum of the reduced sample displays an identical signal intensity of the sample at $g = 1.94$ which was approximately 50% of that of the native protein of the identical metal concentration. In Figure 6, the NADPH–cytochrome *c* reductase activities are compared with the native protein, the sample from the reaction mixture containing Fe and Co, and the sample with Co only. The results showed that the sample with Fe and Co exhibited approximately 50% of the activity of the native protein. The sample with Co alone had negligible activity. In these experiments, the total metal

concentrations were set constant for comparison. Additionally, the isolated products from the sample with Fe and Co were revealed to contain approximately 50% Fe and 50% Co, as shown by metal analysis.

Discussion

From the results presented here, it is clear that when 6 M urea is present, Co and Ru along with the native metal, iron, are incorporated into the apoprotein. Unfolding of the peptide chain is a requirement for the Co and Ru incorporation, whereas this is not required for the Fe incorporation. Under the conditions where the peptide chain is unfolded, Fe and Co can be simultaneously incorporated into the protein at equimolar quantities. Yet, Ru is not incorporated in the presence of iron. The unfolded protein lacks the ability to distinguish Co from Fe, but it is still capable of differentiating Ru from Fe. Of importance is the fact that there was no formation of a mixed complex containing one atom each of Fe and Co per molecule of the protein. In other words, Fe and Co are incorporated as pairs of Fe–Fe and Co–Co into separate molecules. This behavior of the metal derivatives is in contrast to that with selenium incorporation. When both sulfur and selenium are present in the reaction mixture, we were able to detect a mixed sulfur–selenium complex, $Fe_2SeS(SR)_4$ (Mukai *et al.*, 1973, 1974).

Although the coordination structure of the Co derivative has not been finally determined, the line of evidence collected here supports that the complex has a similar structure to the native iron–sulfur chromophore; (1) chemical analyses of cobalt and labile sulfur, (2) a large molar extinction coefficient, (3) pH-stability profile, (4) mercurial titration curve, and (5) diamagnetic properties at 77°K. Dennard and Williams (1966) proposed spectrophotometric diagnosis of the symmetry of Co(III) complexes. Octahedral Co(III) complexes having H_2O as ligands have their main peak at about 500 nm, while the peak for tetrahedral complexes with H_2O ligands would be found at about 600 nm. In a tetrahedral complex, the band is usually split in a characteristic way and the molar extinction coefficient is about 1000, while for the octahedral complex, the coefficient does not exceed 50.

The 470-nm band of the Co protein appears incompatible with the formation of an octahedral Co(II) complex. This band was dissimilar to those of the tetragonal Co(III) complex $(Co(NH_3)_2(H_2O)_4$ having a coefficient of 9 at 500 nm) or a trigonal bipyramid Co(II) complex. Furthermore, the 470-nm band appears unlikely to be an octahedral Co(III) complex with sulfur ligands, since the forbidden band ($^1A_1 \rightarrow {}^3T_1$) was not seen at the near-infrared region (≥700 nm).

Instead, we observed a d–d transition band of the Co-substituted protein at 1430 nm. If one accepts the fact that the Co-substituted protein has a tetrahedral symmetry of high-spin d^7 configuration, this band would correspond to the transition $^4A_2 \rightarrow {}^4T_1$ (F). In a tetrahedral field, only the high-spin state is possible for Co(II) complexes. This assignment is supported by the fact that a typical tetrahedral Co(II) complex, $[CoCl_4]^{2-}$, has the transition of $^4A_2 \rightarrow {}^4T_1$ (F) at 1390 nm. The band which belongs to the highest d–d transition $^4A_2 \rightarrow {}^4T_1$ (P) would be around 520 nm, as predicted by the weak crystal field model. Unfotunately, this d–d band was largely overlapped by the intense 470-nm band. Additionally, the model predicts that the lowest d–d band would be around 3000 nm ($^4A_2 \rightarrow {}^4T_2$).

Under the same assumption, the *Dq* calculated from the 1430-nm band is 390 cm^{-1}. This value is comparable to *Dq* values of $[Co(H_2O)_6]^{2+}$ and $[CoCl_4]^{2-}$ of 950[1] and 360 cm^{-1}, respectively. In this context, the metal in the protein most likely to be a high-spin Co(II) in tetrahedral asymmetry. Yet, the final assignment has to await further elucidation. In particular, the possibility of pentacoordinated and planar Co(II) complexes has not been excluded by the present experiments.

Since our titration experiments with the mercurial reagent support that the cobalt atom is bound to four sulfur atoms, an intense charge transfer band between cobalt and sulfur atoms should be present in the visible region. Thus, we favor to interpret the band at 470 nm with the extinction coefficient of 2200 M^{-1} cm^{-1} as a charge transfer band. If the 470-nm band is the d–d transition of $^4A_2 \rightarrow {}^4T_1$ (P), the *Dq* will be 570 cm^{-1}, which is too large for Co–S complexes.

Another intriguing feature is the enzymatic activity of those derivatives: the selenium-replaced protein has a comparable activity (approximately 70%) to that of the native protein, while the Co- and Ru-substituted proteins have no enzymatic activity. We believe that this difference in enzymatic activity may be partially interpreted in terms of differences in oxidation–reduction potentials. The Co- and Ru-substituted proteins which resist the reduction by NADPH-diaphorase or dithionite presumably have more negative potential than that of the native protein (E_m = −274mV, at pH 7.0) (Huang and Kimura, 1973).

As a conclusion, the ability to select iron among other metal ions appears to be intrinsic to the amino acid sequence and the resulting three-dimensional conformation of the adrenal iron–sulfur protein. This requirement is absolute to produce its enzymatic activity.

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[1] Since *Dq* (tet) = $\frac{4}{9}$*Dq* (oct), the value for tetrahedral symmetry will be about 400 cm^{-1}. The *Dq* = 390 cm^{-1} would be reasonable for the Co–S complex.