Near-Infrared Circular Dichroism of an Iron–Sulfur Protein. d → d Transitions in Rubredoxin

William A. Eaton* and Walter Lovenberg

Contribution from the Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, and the Experimental Therapeutics Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014.
Received April 29, 1970

Abstract: The iron atom in the protein rubredoxin is known from X-ray studies to be roughly tetrahedrally coordinated to the mercaptide sulfurs of four cysteinyl residues. In the wavelength range 1.1–1.8 μ ferrous rubredoxin exhibits a single broad *electronic* absorption band with a maximum extinction coefficient of 120 M^{-1} cm^{-1} at 1.6 μ. Circular dichroism measurements reveal a very large anisotropy factor for the band of about 0.05. The energy, intensity, band width, and anisotropy factor indicate that the 1.6-μ band arises from the *magnetic*-dipole allowed $^5E \rightarrow {}^5T$ transition predicted for a high-spin ferrous ion in a tetrahedral ligand field. On the other hand, these results may be taken as independent evidence for the approximate tetrahedral bonding of the ferrous ion in rubredoxin. To a first approximation Dq is -625 cm^{-1}, thereby establishing the position of a mercaptide sulfur in the spectrochemical series for the first time in any transition metal complex. A simple inexpensive method for measuring circular dichroism is described, which employs a double beam recording spectrophotometer and quarter-wave retardation plates.

Although there have been a large number of investigations of the electronic structure of the central metal in iron-containing proteins, the identification of d → d optical transitions has remained elusive. The only previous study has been reported recently by Eaton and Charney for the heme proteins.[1] The ligand-field spectrum is a particularly important part of the electronic spectrum to understand, since the optical parameters can be directly connected to theoretical descriptions of the chemical bonding. In this work we report the identification of the $^5E \rightarrow {}^5T$ ligand field transition of ferrous rubredoxin from the bacterium *Clostridium pasteurianum* by absorption and natural circular dichroism measurements in the wavelength range 1.1–1.8 μ. A simple method of measuring circular dichroism for very optically active materials, using a Cary-14 recording spectrophotometer, is described. In addition to providing new important experimental information for understanding the iron–sulfur bonding in rubredoxin, the results of this optical study should serve as a basis for investigations of the ligand-field spectra of the more complex iron–sulfur proteins.

Rubredoxin from *Clostridium pasteurianum* is a small iron–sulfur protein consisting of a single polypeptide chain of 55 amino acid residues with one iron atom per molecule.[2] Although its biological function is not yet established, it is assumed to act as an electron-transfer enzyme, since it interacts with many redox systems of this organism.[3] Furthermore, a rubredoxin from *Pseudomonas oleovorans* has been shown to participate in hydrocarbon oxidation.[4] Figure 1 shows a schematic drawing of the iron–sulfur complex in rubredoxin from *Clostridium pasteurianum*.[5,6] Chemical evidence indicated that the iron is coordinated to the mercaptide sulfurs of the only four cysteinyl residues of the polypeptide.[7,8] This has been confirmed on oxidized rubredoxin by the recent 2.5-Å resolution X-ray crystallographic investigation of Herriott, *et al.*, which further shows that the coordination of the iron is roughly tetrahedral.[9] The tetrahedral geometry apparently persists upon one-electron reduction.[10] The optical spectroscopic results presented here may be taken as independent evidence for the approximately tetrahedral coordination of the iron in reduced rubredoxin.

Rubredoxin has been investigated by a variety of other physical methods, including magnetic susceptibility, proton magnetic resonance, Mössbauer spectroscopy,[11] and electron spin resonance.[7] These results, including the optical data from this work, are consistent with considering the state of the iron in oxidized and reduced rubredoxin as high-spin ferric and high-spin ferrous, respectively. In ferric rubredoxin (^6A ground state) all d → d transitions of the iron are spin forbidden and are obscured by the relatively much more intense charge-transfer spectrum which begins at about 12,500 cm^{-1}. The situation is much more favorable for the identification of d → d transitions in ferrous rubredoxin. The ground state of the free

* To whom correspondence should be addressed at the Laboratory of Physical Biology.

(1) W. A. Eaton and E. Charney, *J. Chem. Phys.*, **51**, 4502 (1969); W. A. Eaton and E. Charney in "Structure and Function of Macromolecules and Membranes," B. Chance, T. Yonetani, and C. P. Lee, Ed., in press.

(2) A protein containing sulfur as a ligand for iron, with no porphyrin ligands, is given the general name iron–sulfur protein. An iron–sulfur protein with a characteristic visible and ultraviolet absorption spectrum and containing no "inorganic" sulfide is classified as a rubredoxin. An excellent review of the chemical and physical properties of iron–sulfur proteins is given by J. C. M. Tsibris and R. W. Woody in "Coordination Chemistry Reviews," Elsevier, Amsterdam, in press.

(3) W. Lovenberg and B. E. Sobel, *Proc. Nat. Acad. Sci. U. S.*, **54**, 193 (1965).

(4) J. A. Peterson and M. J. Coon, *J. Biol. Chem.*, **243**, 329 (1968).

(5) All subsequent references to rubredoxin refer to rubredoxin from *Clostridium pasteurianum*.

(6) The partial sequence shown in Figure 1 has been determined by K. McCarthy and W. Lovenberg, unpublished results.

(7) W. Lovenberg in "14th Conference on Proteins of Biological Fluids," H. P. Brugge, Ed., Elsevier, Amsterdam, 1965, p 165.

(8) W. Lovenberg and W. M. Williams, *Biochemistry*, **8**, 141 (1969).

(9) J. R. Herriott, L. C. Sieker, L. H. Jensen, and W. Lovenberg, *J. Mol. Biol.*, **50**, 391 (1970).

(10) L. H. Jensen, private communication.

(11) W. D. Phillips, M. Poe, J. F. Wieher, C. C. McDonald, and W. Lovenberg, *Nature (London)*, **227**, 574 (1970).

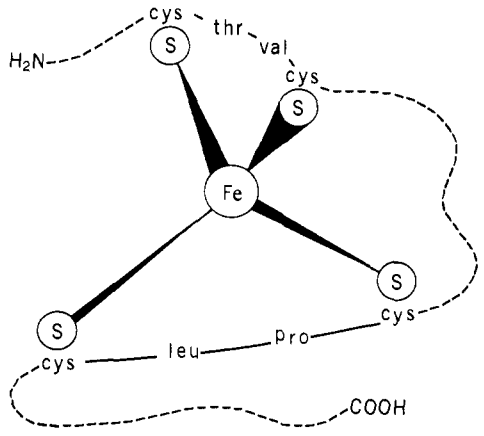

Figure 1. Schematic structure of the iron–sulfur complex in rubredoxin from *Clostridium pasteurianum*.

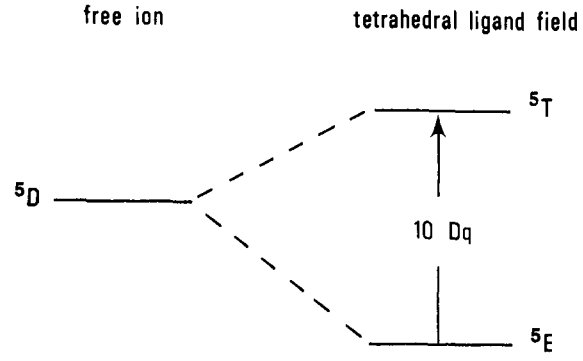

Figure 2. Quintet states of a high-spin d^6 ion in a weak tetrahedral ligand field.

ferrous ion is ^5D which is split in a weak tetrahedral ligand field into a ^5E and a ^5T state (Figure 2).[12] The $^5E \rightarrow {}^5T$ transition is the only spin-allowed ligand field transition and is expected in the near-infrared where there is no interference from charge-transfer bands, which begin at about 25,000 cm^{-1}. This d → d transition arises from one-electron promotions from a mainly $e(d_{x^2-y^2},d_{z^2})$ iron orbital into a mainly $t(d_{xy},d_{xz},d_{yz})$ iron orbital. As a first approximation, the energy at which this transition appears is equal to the energy difference between the triply degenerate $t(d_{xy},d_{xz},d_{yz})$ and the doubly degenerate $e(d_{x^2-y^2},d_{z^2})$ iron orbitals, and is known as $10Dq$ or Δ.

Experimental Section

Rubredoxin was isolated and purified according to the method described by Lovenberg and Williams,[8] modified to prepare the relatively large quantity of protein required for this investigation, about 500 mg. The spectral measurements were carried out using D_2O as the solvent. H_2O at the 25-mm pathlengths required for our measurements is essentially opaque at wavelengths longer than 1.3 μ, whereas D_2O is sufficiently transparent at wavelengths shorter than 1.8 μ. HOD is more highly absorbing than D_2O below 1.8 μ, so that the following procedures were carried out to remove the exchangeable hydrogens, especially the hydrogens of water which are tightly bound to the protein. Ferric rubredoxin was soaked for 24–48 hr in D_2O and then lyophilized. This was repeated several times and during one of these exchange cycles ferric rubredoxin was reduced with sodium dithionite and, after several hours, was allowed to reoxidize in air.

(12) C. J. Ballhausen, "Introduction to Ligand Field Theory," McGraw-Hill, New York, N. Y., 1962.

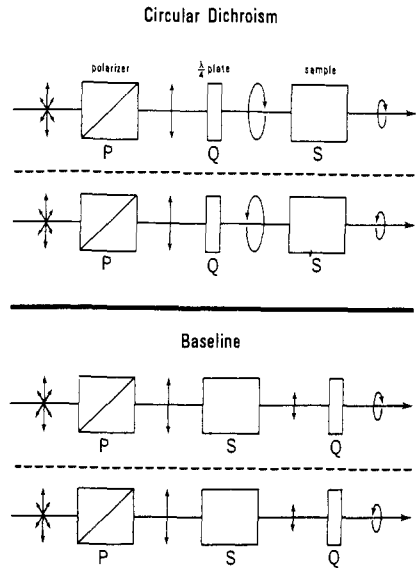

Figure 3. Block diagram of the attachment used for measuring circular dichroism on a Cary 14 recording spectrophotometer. A 0–0.1 optical density unit slide wire was employed in the measurements.

The absorption and circular dichroism measurements were carried out on a Cary 14 double beam recording spectrophotometer. Figure 3 shows a block diagram of the attachment constructed for the circular dichroism measurements, showing the two configurations of the components in the cell compartments used in recording the apparent circular dichroism and the base line. We shall refer to these two configurations as the CD and B configurations, respectively. In the CD configuration light from a tungsten source is plane polarized by a Glan prism, P, circularly polarized by a mica retardation plate, Q, passed through the sample S, and then into the monochromator. In one cell compartment right circularly polarized light is absorbed by the sample, whereas in the other cell compartment left circularly polarized light is absorbed by the sample. In order to obtain the base line, the B configuration is used. The retardation plates are placed after the samples (no other component is moved), so that plane-polarized light now enters the samples. There can of course be no difference in the absorption of *plane*-polarized light for perfectly matched solution samples. The difference between the optical densities recorded by the instrument for the CD and B configurations arises from the difference in optical densities of the sample for right and left circularly polarized light, *i.e.*, the circular dichroism of the sample.[13]

A major virtue of this rather simple and relatively inexpensive method for measuring circular dichroism is the way in which the base line is obtained. By not replacing the sample with solvent, the slit and gain conditions of the instrument are virtually identical in the CD and B configurations. Furthermore, it can easily be shown that the error due to mismatch of the samples in the two cell compartments, *e.g.*, from differences in pathlength and/or concentration, is negligible. Identical solutions are placed in each cuvette which have the same pathlengths to better than 1 part in 1000. In any event, the mismatching of samples leads to a measured circular dichroism whose magnitude is *always smaller* than the true circular dichroism for the more highly absorbing of the two samples.

Abu-Shumays and Duffield have shown that errors arising from scattering and deviation from perfect circular polarization contribute to *lowering* the magnitude of the measured circular dichroism compared to the true value.[14] At the long wavelengths of these measurements, 1.1–1.8 μ, scattering is minimal. The mica plates

(13) In the B configuration the samples will in general rotate the plane of polarization and produce elliptically polarized light, so that for a given wavelength the polarization state of the light entering the monochromator is slightly different in the CD and B configurations. Because of the sensitivity of the monochromator to the polarization state of the light, the difference between the optical densities recorded in the CD and B configurations may contain a small contribution from these optical rotation and ellipticity effects.

(14) A. Abu-Shumays and J. J. Duffield, *Anal. Chem.*, 38, 29A (1966).

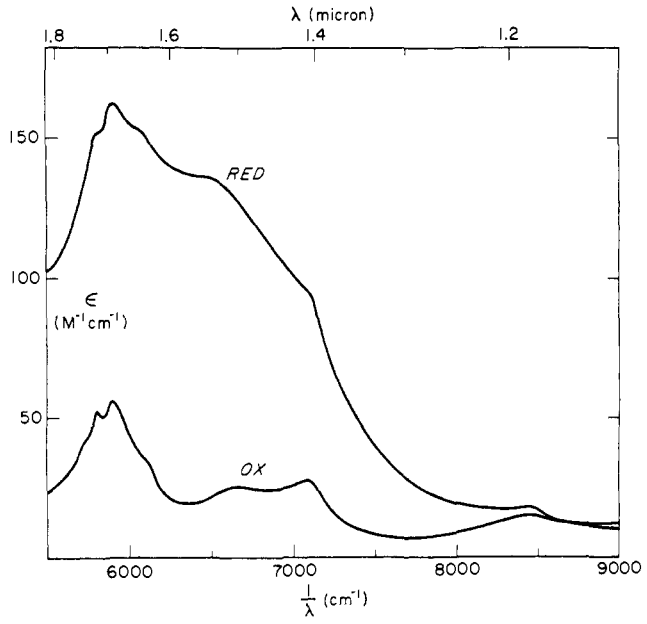

Figure 4. Absorption spectra of ferrous and ferric rubredoxin in D_2O, 0.1 *M* Tris buffer, pH 6, room temperature. The upper curve is ferrous rubredoxin and the lower curve ferric rubredoxin. D_2O was used as a reference in obtaining both spectra. Extinction coefficients are based on the value of 8800 M^{-1} cm^{-1} for the 4900-Å band of ferric rubredoxin.[8]

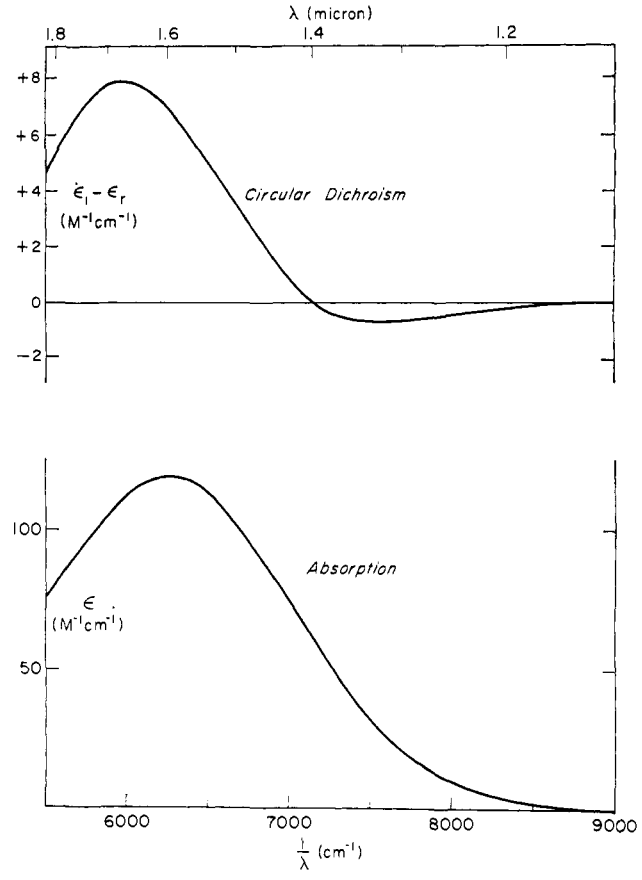

Figure 5. Circular dichroism and absorption of ferrous rubredoxin. The upper curve is the circular dichroism spectrum of ferrous rubredoxin in D_2O, 0.1 *M* Tris buffer, pH 6, room temperature. The lower curve is the difference absorption spectrum, ferrous rubredoxin minus ferric rubredoxin. In the circular dichroism measurement the maximum ($OD_l - OD_r$) was +0.07 optical density unit, which is equal to +2.3° ellipticity.

employed (0.28–0.45 μ relative retardation)[15] were sufficiently close to being exactly quarter-wave retarders that the maximum error due to deviation from perfect circular polarization was calculated to be less than 10%.[14]

There are obviously a variety of possible control experiments which can be tried to test the reliability of the measurements. One of the more critical tests was that the "measured" circular dichroism of optically *inactive* samples, such as D_2O to which some H_2O was added, was less than 0.002 optical density unit for samples with a total optical density of up to 2.5.

Results and Discussion

Figure 4 shows the absorption spectra of ferric and ferrous rubredoxin in D_2O from 5500 to 9000 cm^{-1}. In Figure 5 the difference spectrum, ferrous rubredoxin minus ferric rubredoxin, is shown, along with the circular dichroism of ferrous rubredoxin. Ferric rubredoxin gave no measurable circular dichroism in this spectral region.

The absorption spectrum of ferric rubredoxin (Figure 4) arises from *vibrational* overtone transitions of the protein, which show no measurable circular dichroism, as expected.[16] There is also some contribution to the absorption spectrum from vibrational overtone transitions of HOD which was not completely removed in preparing the sample. Upon reduction with sodium dithionite the absorption spectrum is greatly intensified. The striking feature of the absorption spectra is that in spite of the structured absolute spectra of both ferric and ferrous rubredoxin (Figure 4), the difference spectrum (Figure 5) shows a single smooth absorption band with a maximum extinction coefficient of 120 M^{-1} cm^{-1} at about 6250 cm^{-1}. The 6250-cm^{-1} band in the difference spectrum is clearly an *electronic* absorption band of ferrous rubredoxin. The low energy, low intensity, and large band width of the 6250-cm^{-1} band indicate that it arises from the $^5E \rightarrow {}^5T$ transition of the tetrahedrally coordinated high-spin ferrous ion.

Convincing additional evidence for this assignment comes from the circular dichroism measurements in Figure 5. The absorption intensity for all but extremely weak electronic transitions depends on the square of the magnitude of the *electric* dipole transition moment, whereas the rotational strength depends on the dot product of the *electric* and *magnetic* moments.[17] Therefore, inherently *electric* dipole forbidden transitions with large *magnetic* dipole transition moments, such as certain d → d transitions, have the diagnostic property of exhibiting weak absorption bands, but relatively intense circular dichroism bands.[18,19] The ratio of the rotational strength to the dipole strength is known as the anisotropy or dissymmetry factor and is given approximately by $(\epsilon_l - \epsilon_r)/\epsilon$. Mason has suggested that an anisotropy factor greater than 0.01 may be expected for *magnetic*-allowed transitions.[19,20] The anisotropy factor for the 6250-cm^{-1} band of ferrous rubredoxin is extremely large, about 0.05, as predicted

(15) The mica plates were a kind gift from Dr. Albert Makas of the Polaroid Corporation.

(16) C. W. Deutsche and A. Moscowitz, *J. Chem. Phys.*, **49**, 3257 (1968).

(17) E. U. Condon, *Rev. Modern Phys.*, **9**, 432 (1937).

(18) W. Moffitt, *J. Chem. Phys.*, **25**, 1189 (1956).

(19) S. F. Mason, *Quart. Rev., Chem. Soc.*, **17**, 20 (1963).

(20) The criterion of a large anisotropy factor was used by Eaton and Charney for distinguishing d → d transitions from weak ligand and charge-transfer transitions in the heme proteins.[1]

for the inherently *magnetic*-allowed $^5E \rightarrow {}^5T$ transition.[21] This anisotropy factor was calculated from the absolute magnitudes of the rotational strength and represents a *minimum* value, since almost all sources of error in the circular dichroism measurement lead to measured values which are smaller than the true value (see Experimental Section). Also there is some cancellation of rotational strength due to overlapping of positive and negative circular dichroism bands. The circular dichroism spectrum shows no structure, again reflecting the lack of any contribution to the measured circular dichroism from the vibrational overtone transitions of the protein.

The identification of the $^5E \rightarrow {}^5T$ transition provides independent evidence for the approximate tetrahedral geometry of the iron–sulfur complex in ferrous rubredoxin. Of course these spectroscopic results are not *strictly* inconsistent with other geometries, such as octahedral or square planar. However, the energy of the transition appears to be too low and the intensity too high ($f = 0.001$).

The single-crystal absorption spectrum of ferric rubredoxin in plane-polarized light exhibits highly (linearly) dichroic charge transfer bands,[22] indicating that the local symmetry of the iron is less than tetrahedral.[23] The electron spin resonance spectrum of ferric rubredoxin also indicates that the local environment of the iron is less than cubic.[7] The lowering of the symmetry from tetrahedral leads to the removal of at least the threefold spatial degeneracies and consequently the possibility of observing more than one ligand-field band. In fact, although only a single ligand-field absorption band of ferrous rubredoxin is clearly resolved, two circular dichroism bands of opposite sign are seen (Figure 5). It is of course possible that there are other components of the $^5E \rightarrow {}^5T$ transition at energies lower than 5500 cm^{-1} where we are unable to make measurements at present because of high D_2O absorption. However, the rather symmetric shape of the absorption and circular dichroism spectra suggests that, unless there is a very large distortion from tetrahedral symmetry, all the components of the $^5E \rightarrow {}^5T$ transition are contained in the 6250-cm^{-1} band. Therefore, we shall consider -625 cm^{-1} as the average value for the tetrahedral splitting parameter, Dq.[24]

This is apparently the only known value of a ligand-field splitting parameter for a mercaptide sulfur (RS^-) in any transition metal complex.[25] In $FeCl_4^{2-}$ Dq was found to be -400 cm^{-1}.[26] Other values come from optical measurements on the ferrous iron substituted at a tetrahedral site in single crystals of CdTe, ZnS, and $MgAl_2O_4$, where Dq was found to be -248, -340, and -447 cm^{-1}, respectively.[27] Dq for the cyanide ion, calculated from $^4/_9$ of the octahedral value for $Fe(CN)_6^{4-}$, is -1400 cm^{-1}.[28] It appears, then, that the cysteine sulfurs of rubredoxin belong roughly in the middle of the spectrochemical series. On the other hand, since sulfur is generally a weak-field ligand,[28] it is attractive to consider the possibility that constraints imposed by the protein conformation are somehow responsible for increasing the ligand field strength of the cysteine sulfurs in rubredoxin above the "normal" value.

Since many iron–sulfur proteins contain "inorganic" sulfide ($Dq = -340$ cm^{-1})[2,27] as well as cysteine sulfur, as a ligand, a tetrahedral ligand field surrounding the iron in these proteins should be weaker than in rubredoxin. This conclusion assumes that only sulfurs are directly bonded to the iron and that the "inorganic" sulfide is bonded as S^{2-}, a fact which has not yet been clearly established.

Acknowledgment. We wish to thank Dr. Elliot Charney for helpful discussion, Mr. Charles Lohr for assistance in constructing the circular dichroism attachment, and Mrs. Eleanor Bruckwick for assistance in preparing the rubredoxin.

(21) The anisotropy factor for the charge-transfer bands is about ten times less, as would be expected for inherently *magnetic*-forbidden transitions.

(22) W. A. Eaton and W. Lovenberg, unpublished results.

(23) A chromophore belonging to a cubic point group is a spherically symmetric absorber of plane-polarized light.

(24) Dq carries a minus sign indicating the inversion of the d orbitals in going from an octahedral to a tetrahedral ligand field.

(25) S. E. Livingstone, *Quart. Rev., Chem. Soc.*, **19**, 386 (1965).

(26) C. Furlani, E. Cervone, and V. Valenti, *J. Inorg. Nucl. Chem.*, **25**, 159 (1963).

(27) G. A. Slack, F. S. Ham, and R. M. Chrenko, *Phys. Rev.*, **152**, 376 (1966).

(28) C. K. Jorgensen, "Absorption Spectra and Chemical Bonding in Complexes," Pergamon Press, Oxford, 1962.