Analysis of the Iron–Sulfur Cluster of Aconitase by Natural and Magnetic Circular Dichroism†

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ABSTRACT: We have examined the iron–sulfur cluster of aconitase, a high-potential iron–sulfur protein, by absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectroscopy. The MCD spectrum of unactivated aconitase, which is presumably oxidized, is similar to those of reduced two iron–two sulfide ferredoxins but distinct from the MCD of known four iron–four sulfide proteins. The magnitude of the natural CD of unactivated aconitase also suggests the absence of four iron–four sulfur clusters. Reduction of the enzyme with dithionite and activation with the cysteine–ascorbate–ferrous ion activation mixture generate spectra which are significantly different from those of any iron–sulfur protein seen to date. We interpret these results as indicating that aconitase does not contain a four iron–four sulfur cluster generally thought to be characteristic of high-potential iron–sulfur proteins. It could contain a two iron–two sulfur center or some other center such as a cyclic three iron–three sulfur center.

Aconitase is a member of the Krebs cycle system of enzymes which catalyzes the interconversion of the three tricarboxylic acids, citric acid, *cis*-aconitic acid, and (+)-isocitric acid (Glusker, 1971). Aconitase from pig heart contains iron and sulfide (Kennedy et al., 1972). Ruzicka & Beinert (1978) have demonstrated that aconitase and the soluble "high-potential" type iron–sulfur protein from beef heart mitochondria are indistinguishable. While the relationship between the high-potential character and enzymatic function is unclear at present, aconitase is now the only high-potential iron–sulfur protein with a known function.

The crystal structures of many of the simpler iron–sulfur proteins have been determined (Orme-Johnson, 1973), and they may be classified into several types on the basis of their active centers. These are (1) a single iron atom bonded to the protein by four cysteine sulfurs (e.g., rubredoxins), (2) clusters containing two iron and two labile sulfur atoms coordinated to four cysteine residues (e.g., plant type ferredoxins), and (3) cubic clusters composed of four iron and four bridging sulfides bonded by the irons to four cysteine residues (e.g., bacterial ferredoxins and high-potential iron–sulfur proteins). Recently Stout et al. (1980) have described an additional type of cluster in *Azotobacter vinelandii* ferredoxin which is composed of three iron and three sulfurs alternating in a planar cyclic structure. This structure is presumably bound by its irons to six cysteines of the protein. There are also iron sulfide cluster proteins which contain other metals in place of an iron (Wolff et al., 1978; Lovenberg, 1977).

All proteins which contain iron sulfide clusters exhibit broad absorption bands extending through the near-ultraviolet and visible region into the near-infrared. Circular dichroism indicates that the longer wavelength absorption bands are primarily d ← d transitions (Eaton et al., 1971) while the more intense bands at shorter wavelengths are charge-transfer transitions. The lack of distinctive structure in the absorption spectra of iron–sulfur proteins makes it difficult to determine the cluster type solely from absorption measurements. The CD[1] and MCD spectra of iron–sulfur proteins contain more structure than the unpolarized absorption spectra. Stephens et al. (1978a,b) pointed out cluster-specific features of the MCD spectra. Thus, we have subjected aconitase to analysis by absorption, CD, and MCD spectroscopy for the purpose of characterizing its iron–sulfur cluster.

Materials and Methods

Aconitase from pig heart was isolated in its inactive, oxidized form as previously described (Kennedy et al., 1972) and stored frozen in 15 mM Tris–tricarballylate, pH 7.8. Tricarballylate, which is a competitive inhibitor of the enzyme (Gawron & Jones, 1977), was used to stabilize the enzyme. Spectroscopic analyses of aconitase were done at 4.0 mg/mL in this same buffer. Dithionite-reduced enzyme was produced by the addition of a 10-fold molar excess of sodium dithionite dissolved in the tricarballylate buffer. Aconitase at 4.0 mg/mL was activated in tricarballylate buffer containing 6 mM ascorbate, 2 mM cysteine, and 1 mM ferrous ammonium sulfate (Gawron et al., 1974). All solutions were deoxygenated prior to use by bubbling nitrogen through them. Spectroscopic analyses were done with samples sealed under nitrogen.

Absorption spectra were measured with a Cary Model 118C spectrophotometer (Varian Associates, Palo Alto, CA). Natural and magnetic circular dichroism were measured with a polarization and emission spectrometer which has been described elsewhere (Sutherland, et al., 1976). MCD spectra were measured at a magnetic field strength of 1.1 T. Both the spectrophotometer and the polarization and emission spectrometer were operated under the control of a computer (Tektronix, Beaverton, OR, Model 4051). The interfaces between the computer, the spectrophotometer, and the P/E spectrometer have been described elsewhere (Sutherland & Boles, 1978; Sutherland et al., 1980). Spectra were stored on flexible disks for post-experimental analysis. Permanent graphs of original and deconvoluted spectra were plotted on a Versatec (Santa Clara, CA) Model 1200A printer/plotter.

The CD scale of the P/E spectrometer was calibrated with a solution of *d*-10-camphorsulfonic acid (Aldrich, Milwaukee, WI) by assuming the ratio of $\Delta A_{CD290.5nm}/A_{285nm}$ equal to 0.068 (Chen & Yang, 1977). The strength of the magnetic field applied to the sample was measured with a gauss meter (RFL Industries, Boonton, NJ, Model 750D).

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[1] Abbreviations used: CD, circular dichroism; MCD, magnetic circular dichroism.

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In CD and MCD the measured parameter is the difference between the absorbance of left and right circularly polarized light which we denote as ΔA. This parameter, which like optical density is dimensionless, is related to ellipticity by the equation $A = \theta/33$. The measured value of ΔA is the sum of the contributions of natural and magnetic components. Thus, using Beer's law we write

$$\Delta A = \Delta\epsilon_{CD}[\text{Acon}]d + H\Delta\epsilon_{MCD}[\text{Acon}]d \qquad (1)$$

where ΔA is the total circular dichroism of the sample, [Acon] is the molar concentration of the protein, d is the optical path through the sample (cm), H is the intensity of the magnetic field (tesla), and $\Delta\epsilon_{CD}$ and $\Delta\epsilon_{MCD}$ are respectively the CD and MCD differential extinction coefficients. Holmquist & Vallee (1978) have presented conversion factors for other systems of units which have been used to describe CD and MCD.

Measurement of the CD and MCD of a sample requires that the spectrum of the sample and of the reference solution be recorded both with the magnetic field on and then with the field off (i.e., $H = 0$). Appropriate reference spectra were also recorded with the field on and off and subtracted from the corresponding spectra of the sample. For more details of the deconvolution procedure, see Sutherland et al. (1974) or Holmquist & Vallee (1978). The deconvolution required to convert the four experimental spectra into spectra of $\Delta\epsilon_{CD}$ and $\Delta\epsilon_{MCD}$ were performed by the computer.

Results and Discussion

An attempt to characterize the iron–sulfur cluster of a protein on the basis of spectral, and especially MCD, data requires two major assumptions. First, it is assumed that all iron–sulfur proteins containing the same type of cluster will have characteristic spectral properties. According to Stephens et al. (1978a,b), this is the case for two iron–two sulfur clusters and for four iron–four sulfur clusters. In addition, one would need to assume that exemplars of all possible iron–sulfur clusters are available for comparison. This second assumption is less certain, and, therefore, limits conclusions which can be drawn from spectral analyses at present.

The absorption, CD, and MCD spectra of unactivated aconitase are shown in Figure 1. Below 300 nm the absorption spectrum is dominated by the absorption of aromatic amino acids. Information about the iron–sulfur complex is obtained from longer wavelengths. The MCD is smaller than the natural CD for the magnetic field strength used in these experiments and only slightly greater than the limit for detectability according to the criterion of Sutherland (1978). There are clear differences between these spectra and those of other iron–sulfur proteins. The features of the spectra which distinguish them from those of proteins which contain four iron–four sulfur clusters are the shape of the MCD and the relative magnitudes of the CD and MCD. The MCD spectra of the three four iron–four sulfur cluster proteins studied by Stephens et al. (1978a,b) are positive for all wavelengths above 330 nm. The MCD consists of a series of peaks or shoulders starting near 2000 nm and progressively increasing in magnitude up to a maximum near 400 nm. The MCD then decreases but remains positive (Stephens et al., 1978a,b). This general pattern is independent of oxidization state. In contrast, the MCD of proteins known to contain two iron–two sulfur clusters all go negative at some point between about 350 and 600 nm (Stephens et al., 1978a), as does the MCD of aconitase shown in Figure 1. In addition, the multiple peaks of roughly equal magnitude separated by a pronounced trough in the 400- to 600-nm range and the negative maximum observed for wavelengths less than 400 nm are features observed for reduced

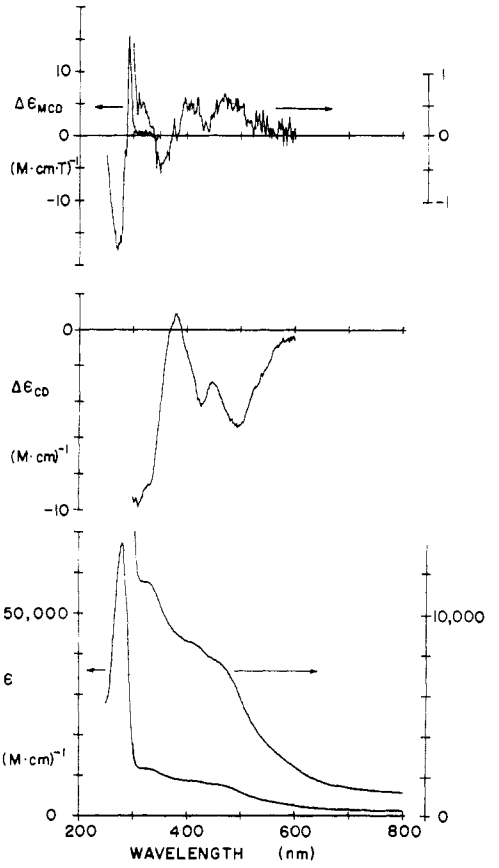

FIGURE 1: Absorption spectrum (lower), CD spectrum (center), and MCD spectrum (upper) of unactivated aconitase. Reversing the direction of the magnetic field inverts the MCD spectrum, indicating that the observed spectrum results from the intrinsic MCD of the sample and is not an artifact associated with deconvolution of the larger signal due to the natural CD.

putidaredoxin, reduced *Spirulina maxima* ferrodoxin (Stephens et al., 1978a), reduced spinach ferredoxin (Sutherland et al., 1972), and reduced adrenodoxin (Thompson et al., 1977), all of which contain two iron–two sulfur clusters. These features of the MCD are thus in contrast to both the MCD of all reported oxidization states of the four iron–four sulfur cluster proteins and also the oxidized states of the two iron–two sulfur proteins mentioned (Stephens et al., 1978a,b). The amplitudes of the positive and negative peaks of the MCD of aconitase are approximately 0.5 $(M\cdot cm\cdot T)^{-1}$, values comparable to those observed for reduced putidaredoxin, reduced *Spirulina maxima* ferredoxin (Stephens et al., 1978a), and reduced spinach ferredoxin (Sutherland et al., 1972). [The latter must be divided by a factor of six to normalize the spectra to a value of 1 T. The data for reduced adrenodoxin (Thomson et al., 1977) were recorded at 6.5 K and are thus not directly comparable.] The peak values of $\Delta\epsilon_{MCD}$ for proteins containing four iron–four sulfur clusters are typically larger by a factor of at least two (Stephens et al., 1978a).

Natural circular dichroism is generally more sensitive to protein structure and hence less diagnostic of cluster type than is MCD (Stephens et al., 1978). However, CD does seem to provide some information on cluster type. For example, the CD spectrum of oxidized two iron–two sulfide proteins typically exhibit a strong ($\Delta\epsilon > 15\ M^{-1}\ cm^{-1}$) positive band between 400 and 500 nm (Dervartanian et al., 1969; Rao et al., 1971; Sutherland et al., 1972; Cardenas et al., 1976; Thompson et al., 1977, Stephens et al., 1978a,b; and references cited therein). The absorption spectra of oxidized two iron–two sulfide proteins also show resolved peaks in the 400–500-nm region. The CD and absorption spectra of aconitase lack these

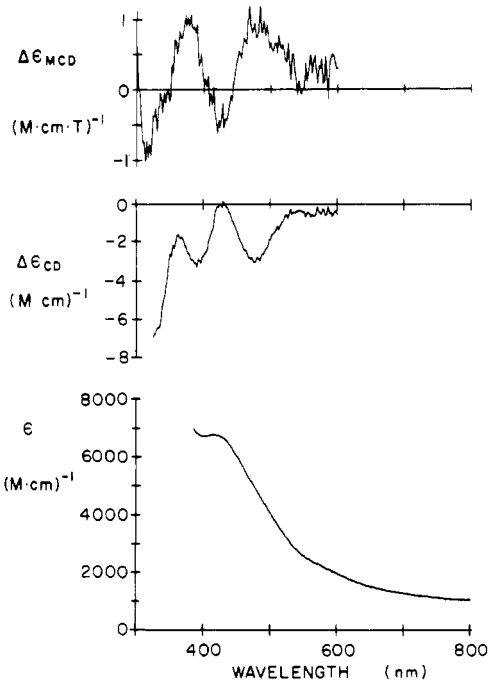

FIGURE 2: Absorption spectrum (lower), CD spectrum (center), and MCD spectrum (upper) of dithionite-reduced aconitase.

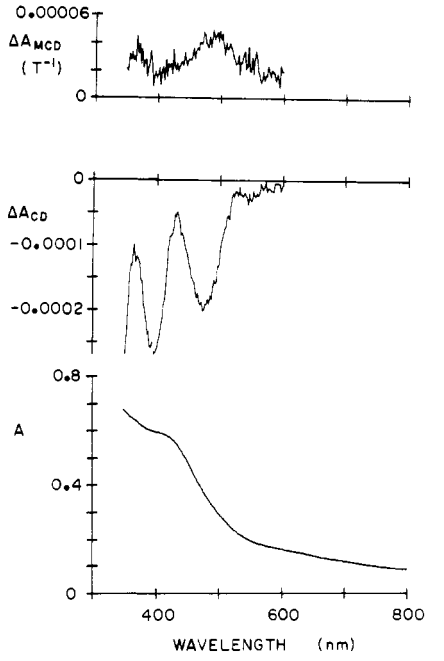

FIGURE 3: Absorption spectrum (lower), CD spectrum (center), and MCD spectrum (upper) of cysteine–ascorbate–ferrous ion activated aconitase.

features. Thus both CD and absorption spectroscopy reinforce the conclusion that if aconitase is a two iron–two sulfide protein, both iron ions are not oxidized (i.e., Fe^{3+}) in the "unactivated" protein.

The absorption CD and MCD spectra of aconitase after treatment with a 10-fold molar excess of sodium dithionite and after treatment with the cysteine–ascorbate–ferrous ion activation mixture (Gawron et al., 1974) are shown in Figures 2 and 3, respectively. The spectra were recorded at a sufficient time after treatment of the unactivated aconitase to permit all chemical reactions to come to equilibrium. The activation solution is colored and exhibits both CD and MCD, thus complicating deconvolution of the spectra attributable to the protein, particularly below 400 nm. Thus, we have presented these spectra in terms of absorbance units rather than molar extinction units. Interference from the activating solution may also have caused base-line shifts in the CD and MCD. The excess dithionite also absorbs below 400 nm. Allowing for small differences which may be due to the activation solution, there is reasonable qualitative agreement between the spectra of dithionite-reduced and "activated" aconitase. Ruzicka & Beinert (1978) reported that aconitase can be at least partially activated by reduction by dithionite; Schloss (1979) reported that activation accompanied reduction by dithiothreitol under argon. Both Ruzicka & Beinert (1978) and Schloss (1979) concluded that "activation" is in reality reduction of the enzyme. However, we (K. Hahm and D. Piszkiewicz, unpublished results) have been unable to regenerate any aconitase activity by reduction with either dithionite or dithiothreitol. Thus, the relation between reduction and activation remains unclear.

Comparisons of the spectra shown in Figures 2 and 3 with those of other reduced iron–sulfur proteins (Stephens et al., 1978a,b) reveals that no consistent correlations can be made. For example, the absorption spectra shown in Figures 2 and 3 are similar to the absorption spectra of several four iron–four sulfur proteins. However, the corresponding MCD spectra are strikingly different. Two possible explanations present themselves. First, the reduced center of activated aconitase may be a fully reduced two iron–two sulfur center (i.e., with both irons formally in the ferrous state). To the best of our knowledge a fully reduced two iron–two sulfur center has yet to be observed in any protein. Furthermore, its transition between oxidized and reduced states would be expected to have a reduction potential lower than that found in two iron–two sulfur ferredoxins. Alternatively, the observed MCD spectra of activated and dithionite-reduced aconitase may represent a center not previously examined by MCD, such as the cyclic three iron–three sulfur cluster (Stout et al., 1980).

The nature of the iron sulfide center of aconitase has been the subject of much study which, to date, has not yielded a concensus. This may reflect significant differences in the enzyme preparations used by the several investigators. Reported values of the iron and sulfide content have varied. Pig heart aconitase was found to contain two iron and three sulfides per molecule of 66 000 daltons in the unactivated form (Kennedy et al., 1972) and three iron and three sulfides per molecule when activated (Gawron et al., 1974). A preparation of this same enzyme was reported (Villafranca & Mildvan, 1971) to contain one iron per molecule of 89 000 daltons, but was significantly less active. Aconitase from *Candida lipolytica* was reported (Suguki et al., 1976) to contain two irons and one sulfide ion as isolated and two ions each of iron and sulfide in the reconstituted enzyme. Ruzicka & Beinert (1978) reported approximately two irons and two sulfides per enzyme molecule of 89 000 daltons, but also raised the possibility that iron and sulfide were lost during purification. If this is the case, reported contents of iron and sulfide would represent minimum values. The iron sulfide center in aconitase has been concluded to be of the cubic, four iron–four sulfide type on the basis of its high-potential character (Ruzicka & Beinert, 1978) and of the binuclear, two iron–two sulfide type on the basis of cluster displacement experiments (Kurtz et al., 1979). The recent discovery by Stout et al. (1980) of a three iron–three sulfur center has led to the suggestion that aconitase may also contain this structure (Emptage et al., 1980). In a note added in proof, Emptage et al. (1980) cite simmilarities between the Mössbauer spectra of beef heart aconitase and two prokaryotic ferredoxins which are believed to have iron–sulfur clusters containing three iron ions.

In view of the conflicting results and conclusions concerning the cluster composition and type of aconitase which have been reported, we are inclined to interpret our spectral characterization of this enzyme with caution. We conclude that aconitase does not contain a four iron–four sulfur center generally thought to be characteristic of high-potential iron–sulfur proteins (Orme-Johnson, 1973; Ruzicka & Beinert, 1978). Aconitase could contain a two iron–two sulfur center which must be fully reduced to have catalytic activity. The enzyme could also contain some other center such as the recently discovered cyclic three iron–three sulfur cluster (Stout et al., 1980). Assuming that the identification of the iron–sulfur cluster of aconitase as being of the three-iron type (Emptage et al., 1980) is substantiated, this paper is the first report of the CD and MCD of such a cluster.

The specific function of the iron–sulfur cluster in the reaction catalyzed by aconitase still remains to be defined. The fact that this enzyme is the high-potential iron–sulfur protein of mitochondria has raised the possibility that the iron–sulfur center may have a regulatory function (Ruzicka & Beinert, 1978). It may serve as a sensor for the oxidation–reduction potential of the environment which is involved in the inactivation of the enzyme. However, the possibility that it is also involved in the catalytic mechanism cannot be discounted. Gawron & Jones (1977) have suggested that a ferrous ion might undergo successive oxidation and reduction in polarizing the double bond of *cis*-aconitic acid prior to hydration. The iron–sulfur cluster of aconitase might conceivably participate in catalysis by a similar mechanism. The high-potential character of aconitase may thus be unrelated to an electron-transport function. Rather, it may be a mechanism for assuring that the iron–sulfide center is kept in the reduced, catalytically active form.

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