Characterization of the Fe-S Cluster in Aconitase Using Low Temperature Magnetic Circular Dichroism Spectroscopy*

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Beef heart aconitase has been studied by low temperature magnetic circular dichroism (MCD) spectroscopy in the wavelength region 300 to 1900 nm. Together with parallel electron paramagnetic resonance and activity measurements, these data enable correlations between Fe-S cluster-type and enzymic activity in aconitase. In samples not exposed to extraneous Fe, the Fe-S cluster in aconitase exhibits the characteristic properties of a 3Fe center in both the as isolated and dithionite-reduced states. On the basis of the detailed form of the low temperature MCD spectra, three types of 3Fe center can be distinguished in biological samples. These are typified by the 3Fe centers in aconitase, Desulfovibrio gigas Fd, and Azotobacter croccocum Fd. In aconitase, maximal enzymic activity is found to be associated with the build-up of [4Fe-4S] clusters in good agreement with the Mossbauer studies of Kent et al. (Kent, T. A., Dreyer, J. L., Kennedy, M. C., Huynh, B. H., Emptage, M. H., Beinert, H., and Münck, E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1096–1100). However, significant catalytic activity (~60%) was obtained by reduction of the 3Fe center with dithionite in the absence of added Fe. The form and intensity of the resultant MCD spectrum are consistent with the majority of the Fe being in the form of reduced 3Fe clusters. The possibility that a reduced 3Fe cluster is capable of promoting partial catalytic activity in aconitase is discussed in light of these results.

Aconitase (citrate (isocitrate) hydrolase, EC 4.2.1.3) catalyzes the stereospecific conversion of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle.

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The requirement for incubation with ferrous ion to promote maximal activity led to the suggestion that the Fe was involved in the catalytic mechanism (1, 2). However, subsequent characterization by analytical and spectroscopic techniques revealed aconitase to be an iron-sulfur protein (3–8). This information together with the observation that activity develops after reduction of the cluster even without added iron (4, 5, 9, 10) has cast doubt on mechanisms involving protein-bound Fe(II) ion at the active site. It has been suggested (9) that the Fe-S cluster could regulate the activity of the enzyme, but the precise mechanism and physiological function of such a role remains unclear.

The Mössbauer and EPR properties of the iron-sulfur center in aconitase as isolated are indicative of a 3Fe-containing iron-sulfur cluster (6). This new class of biological iron-sulfur centers is the subject of a recent review article (11). Since their initial discovery in a ferredoxin (FdI) from Azotobacter vinelandii (13), similar centers have been identified spectroscopically in a range of bacterial ferredoxins (FdII and II from Desulfovibrio gigas (13–15), Thermus thermophilus (16), Methanosarcina Barkeri (17), glutamate synthase from A. vinelandii (12), and hydrogenase from Desulfovibrio desulfuricans (18).

The structural aspects of 3Fe clusters in iron-sulfur proteins are currently an area of controversy. Extended x-ray absorption fine structure data for aconitase (17) and D. gigas FdII (19) indicate an Fe-Fe distance of 2.7 Å, whereas x-ray crystallographic data for the 3Fe center in A. vinelandii FdII indicate an almost planar [3Fe-3S] cluster with an average Fe-Fe distance of 4.1 Å (20). Both sets of results can only be rationalized by invoking two structurally different types of cluster. However, the uniformity of the electronic properties of known 3Fe clusters, as demonstrated by Mössbauer and EPR spectroscopy, argues against this conclusion. Moreover, recent resonance Raman studies for a range of 3Fe-containing iron-sulfur proteins, including those in D. gigas FdII and A. vinelandii FdII, furnish strong evidence for structurally analogous 3Fe clusters with minor differences in the mode of coordination to the protein (45). Vibrational analysis and comparison with inorganic metal-sulfide clusters of known structure point to a cluster of stoichiometry [3Fe-4S]. While the resonance Raman spectrum of aconitase in its inactive state is different in detail from that exhibited by bacterial 3Fe clusters, the pattern of vibrational modes is again indicative of a [3Fe-4S] center, albeit with slightly modified structural...
parameters (46). This core stoichiometry was first suggested as a result of careful analysis of the non-heme iron and acid-labile sulfur content of inactive aconitase. The most recent data indicate non-heme Fe to S²⁻ ratios between 0.66 and 0.74 (7, 46).

It is now firmly established that 3Fe centers of the type seen in D. gigas FeIII are readily generated by oxidative damage to [4Fe-4S] clusters in bacterial ferredoxins (21-23). This has led to speculation that 3Fe clusters may be artifacts produced by oxidative damage during isolation. Unlike the bacterial ferredoxins, aconitase has a well defined and easily measurable activity, making it suitable for testing this hypothesis. In contrast to aerobic isolation procedures, anaerobic isolation yielded aconitase which retained considerable (20-40%) activity prior to activation (9). A further improvement, namely a rapid isolation method (7), yielded enzyme with about 90% of its maximum activity without activation, and such preparations contained iron and labile sulfur in the ratio of 1:1. Mössbauer spectroscopy showed that this preparation contained a [4Fe-4S] cluster, suggesting that the 3Fe center found after oxidative purification is an artifact of the isolation procedure. From Mössbauer experiments on inactive and Fe-activated aconitase it was then possible to rationalize the requirement for extraneous Fe(II) to promote maximal activity. It has been proposed that added Fe(II) ion is incorporated into the iron-sulfur cluster to produce a catalytically active enzyme containing a [4Fe-4S]²⁺ center. The ease of interconversion between 3Fe and 4Fe centers has also been demonstrated for D. gigas FeII (24, 25) and Clostridium pasteurianum Fd (22).

MCD spectroscopy, when performed at temperatures down to 1.5 K, provides a sensitive optical probe for paramagnetic centers in particular. This novel use of MCD arises because paramagnetic chromophores invariably yield temperature-dependent MCD spectra, whereas diamagnetic species yield MCD signals that are independent of temperature and linearly dependent on magnetic field intensity. For iron-sulfur proteins, this technique has proven effective both as a method of cluster-type determination, by examination of the detailed form of the low temperature spectrum (26), and in establishing electronic ground state properties from MCD magnetization curves (15, 21, 26, 28). The technique is particularly useful in monitoring and characterizing the electronic properties of EPR-silent paramagnets such as reduced 3Fe centers (15, 21).

In the present study, we report low temperature MCD spectra and magnetization data for beef heart aconitase together with parallel activity and EPR measurements. The results establish the nature of the iron-sulfur cluster in a variety of aconitase samples, enabling correlations to be made between cluster-type and enzyme activity. In addition the question of the degree of homogeneity among 3Fe centers in iron-sulfur proteins is addressed by comparing the MCD spectra of aconitase with those of other 3Fe cluster-containing proteins.

MATERIALS AND METHODS

Aconitase was isolated from beef heart mitochondria following the procedure of Ruzicka and Beinert (4) except that argon-flushed buffers were used throughout the procedure. Preparation 1 (used to prepare all the derivatives for EPR and MCD studies) underwent a further purification step involving affinity elution of aconitase in 20 mM Hepes at pH 7.5 from CM-cellulose with buffer and 0.5 mM cis-aconitase, as described by Davies and Scopes (29). Analytical assays for non-heme Fe (30) and acid-labile S (31) were performed according to the published procedures. Aconitase activity was assayed at 26°C using a method based on that devised by Rose and O’Connell (32). The assay mixture consisted of (a) 2.92 ml of 100 mM triethanolamine-Cl, 1 mM MgCl₂, 2 mM sodium citrate (pH adjusted to 8.0 using NaOH), (b) 30 μl of 100 mM NADP (pH adjusted to 6.5 using NaOH), (c) 50 μl of isocitrate dehydrogenase (Sigma type IV) at a concentration of 10 mg/ml, 10-μl aliquots of the aconitase samples used for MCD and EPR experiments were added anaerobically to 1 ml of pH 7.5, 20 mM Hepes buffer or activating solution and incubated for 30 min at room temperature. The activating solution contained 0.5 mM FeSO₄ and 0.5 mM diethiothreitol and was used to assess maximal activity. 40-μl aliquots of the resulting solutions were subsequently added to 3 ml of aerobic assay mixture in a 1-cm absorption cuvette spectrophotometrically monitored at 26°C. The production of NADPH was measured as a function time by monitoring absorbance at 340 nm. The activity is expressed in terms of micromoles of NADPH produced per min per mg of aconitase.

All samples contained 5% v/v ethylene glycol to obtain optical quality glasses on freezing MCD samples. The presence of 50% v/v ethylene glycol had no effect on the activity, UV-visible absorption, or CD spectra of aconitase. The CD spectra of frozen samples of all derivatives at 4.2 K bore close resemblance to the published room temperature spectra (8) giving no indication of any conformational changes on freezing.

EPR spectra were recorded using a Bruker ER-200D spectrometer fitted with an Oxford Instruments ESR-9 continuous flow cryostat. The spectra were quantified using the published procedures (33, 34) with 1 mM CuEDTA as standard under nonsaturating conditions.

MCD measurements in the visible and near IR regions were performed using the apparatus and methods previously described (26, 35) and are expressed as Δν = ν₁ - ν₂. The magnetic fields used are indicated in the figure legends. Aconitase concentrations are based on determination of total protein concentration by the method of Lowry et al. (36), modified to include copper in the determination of sulfhydryl content. Magnetic fields used are indicated in the figure legends. Aconitase concentrations are based on determination of total protein concentration by the biuret method.

RESULTS AND DISCUSSION

Table I shows UV-visible absorption, activity, and analytical data for two different aconitase preparations used in the EPR and MCD studies. While others were investigated, these preparations represent extremes of absorbance ratios and activity for the isolated enzyme. In accord with the analytical data recently presented by Beinert et al. (7), aconitase samples yielded Fe/S²⁻ ratios between 0.62 and 0.83. This is consistent with an iron-sulfur core stoichiometry of [3Fe-4S]²⁻. Judging by the Fe analyses all preparations contained a protein impurity deficient in Fe. Preparation 1 had undergone further purification using affinity chromatography, as described under "Experimental Procedures." The absorbance ratios and Fe content are consistent with the lowest percentage of protein impurity in this preparation. No impurity could be detected in this preparation by SDS-gel electrophoresis.

Four distinct chemical derivatives of aconitase were investi-
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**Table II**
Summary of activity and EPR data for aconitase samples used in MCD experiments

| Sample preparation* | Activity* | EPR Spectral details | Spins/3Fe |
|---------------------|-----------|----------------------|-----------|
| Aerobically isolated | 9         | Almost isotropic signal centered at \( g = 2.01 \) | 0.93      |
| Dithionite-treated  | 66        | No EPR signals       |           |
| 10-fold excess incubated for 10 min | 66        | No EPR signals       |           |
| 20-fold excess incubated for 30 min | 66        | No EPR signals       |           |
| Fe(II) ion-treated | 97        | No EPR signals       |           |
| 10-fold excess incubated for 30 min | 97        | Rhombic signal, \( g_1 = 2.05, g_2 = 1.91, g_3 = 1.84 \) | 0.05 to 0.1 |
| Fe(II) ion plus dithionite-treated 10-fold excess incubated for 30 min of Fe(II) ion incubated for 30 min | 97        | Rhombic signal, \( g_1 = 2.05, g_2 = 1.91, g_3 = 1.84 \) | 0.05 to 0.1 |

*All aconitase derivatives were prepared using aconitase preparation 1 and include 50% v/v ethylene glycol.

**Activity is expressed as a percentage of maximal activity. In the presence of 50% v/v ethylene glycol, maximal activity = 12.42 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\).**

The redox potentials, as determined by potentiometric EPR spectroscopy distinguishes three types of oxidized 3Fe clusters. These results, along with those obtained by potentiometric titrations, also divide the 3Fe clusters in sets with high (~50 to ~150 mV) and low (~450 mV) midpoint potentials for aconitase, *D. gigas* FdII and *A. croococcus* Fd, respectively. A similar classification can be made using resonance Raman spectroscopy, which furnishes a more direct probe for the structural aspects of the Fe-S framework. The archetypal centers on the basis of resonance Raman are in aconitase, *A. vinelandii* FdI, and *D. gigas* FdII. However, aside from some diversity in the cysteinyl coordination and minor differences in the structural parameters for the aconitase cluster in particular, all three are considered to contain a common (3Fe-4s) center. *A. vinelandii* FdI and *A. croococcus* Fd are identical in terms of their redox properties, absorption, and EPR characteristics.

Measurement of the low temperature MCD spectrum for an isolated aconitase into the near-IR region reveals electronic transitions out to 2000 nm, as seen in Fig. 4. This is a surprising observation since Mössbauer spectroscopy identifies oxidized 3Fe clusters as containing three high spin Fe(III) ions in approximately tetrahedral coordination (6, 12, 13). It has been assumed on the basis of studies of the natural CD and MCD of oxidized rubredoxin and oxidized [2Fe-2S]+ clusters that no d-d transitions from the 6A1 ground state of the high spin ferric ion lie at wavelengths longer than 1200 nm. However, such transitions are spin-forbidden both in the single center protein and in the dimeric cluster (38–40). It is possible that such transitions lie at even lower energies than previously suspected, for example, high spin Fe(III) in a tetrahedral lattice of S\(^2\), such as CuGaS\(_2\) or CuAlS\(_2\), appears to have its lowest energy d-d band, \( A_1 \) at ~5000 cm\(^{-1}\) (41). Hence a reappraisal of the assignment of the near infrared electronic spectra of iron-sulfur clusters may be necessary. We have recently confirmed the existence of electronic transitions between 1200 and 2000 nm in a variety of proteins containing oxidized 3Fe clusters.

Fig. 5 compares the low temperature MCD spectra of aconitase preparations 1 and 2 as isolated. In total, MCD spectra were recorded for five distinct aconitase preparations. The redox potentials, as determined by potentiometric EPR or optical titrations, also divide the 3Fe clusters in sets with high (~50 to ~150 mV) and low (~450 mV) midpoint potentials for aconitase, *D. gigas* FdII and *A. croococcus* Fd, respectively.

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Fig. 1. Aconitase (preparation 1) as isolated, 78 mg/ml, 50% v/v ethylene glycol. Upper, room temperature absorption spectrum; Lower, MCD spectra at 1.55 K (---), 4.22 K (-----), 32.0 K (-----). Magnetic field = 4.91 Tesla, pathlength = 1.36 mm.

Fig. 2. MCD magnetization plot for aconitase (preparation 1) as isolated. Conditions of measurement as for Fig. 1, wavelength = 698 nm. Temperatures, 1.61 K (+), 4.22 K (x), 20.0 K (O), magnetic fields between 0 and 4.91 Tesla. Arrow indicates intercept value = 0.49 ± 0.02 k, Boltzmann constant.

Fig. 3. Comparison of MCD spectra for aconitase (preparation 1), Azotobacter croococum Fd, and Desulfovibrio gigas FdII. All samples are as isolated. Conditions: temperature = 1.5 K, magnetic field = 5 Tesla, pathlength = 0.1 mm.

three preparations not shown gave spectra intermediate between those of preparations 1 and 2. In contrast to preparation 1, MCD magnetization curves for preparation 2 were dependent on wavelength. Such behavior is indicative of the presence of at least two chromophoric species. The difference spectrum shown in Fig. 5 identifies the MCD characteristics of the chromophoric impurity. Similar difference spectra, although varying in magnitude, were obtained for all other preparations. The nature of the impurity is not immediately apparent from its low temperature MCD spectrum. It most closely resembles a high spin Fe(III) hemoprotein (42, 43), and the low A_440/A_410 ratio (see Table I) is consistent with this interpretation. There does appear to be a positive correlation between the activity remaining after isolation and the level of impurity, although at present we have no explanation for this behavior. The impurity is not present to a measurable extent in preparation 1 which was subjected to a further purification step involving affinity chromatography. This preparation was used for all subsequent aconitase experiments.

Dithionite-treated Aconitase—The addition of dithionite to anaerobic samples of aconitase causes partial bleaching of the visible absorption spectra. MCD spectra at 4.91 Tesla and temperatures between 1.6 and 34.0 K for aconitase incubated with a 10-fold stoichiometric excess of sodium dithionite for 10 min at room temperature are shown in Figs. 6 and 7 for the regions 300–800 and 800–2000 nm, respectively. Since all the MCD bands display strong temperature dependence, the
Fig. 4. Near IR MCD spectrum of aconitase (preparation 1) as isolated, 78 mg/ml, 50% v/v ethylene glycol. Temperature = 1.55 K, magnetic field = 4.91 Tesla, pathlength = 1.36 mm.

FIG. 4. Near IR MCD spectrum of aconitase (preparation 1) as isolated, 78 mg/ml, 50% v/v ethylene glycol. Temperature = 1.55 K, magnetic field = 4.91 Tesla, pathlength = 1.36 mm.

Electronic transitions must arise from a paramagnetic but EPR-silent ground state. The low temperature MCD spectrum is broadly similar to that of reduced D. gigas FdII (cf. Fig. 2 of Ref. 15). In the latter spectrum two positive peaks are observed at 445 and 485 nm which appear to have coalesced into the single relatively more intense positive peak at 450 nm in the MCD spectrum of reduced aconitase. In addition the peak at 700 nm shows the presence of several additional resolved components compared with that of reduced FdII from D. gigas. The similarity is further established by the presence of the positive peak at 1150 nm, which is also present in the MCD spectra of reduced FdII from D. gigas and several other reduced 3Fe-containing proteins.

The characteristic MCD magnetization properties also confirm the identity as a reduced 3Fe cluster, electronically similar to that in FdII from D. gigas. A representative MCD magnetization plot is shown in Fig. 8. The intercept value, I = 0.18 ± 0.02 for the lowest temperature data and the "nesting" of the curves at different temperatures are both features characteristic of the reduced 3Fe cluster of FdII from D. gigas which have been tentatively interpreted in terms of an $S = 2$ ground state with a predominantly axial distortion leaving an $M_s = \pm 2$ doublet lowest in energy. (For a more detailed discussion see Ref. 15.) Therefore, in agreement with the Mössbauer investigation of Kent et al. (6), we conclude that the Fe-S cluster in aconitase as isolated has the electronic ground state properties of a 3Fe cluster in both the oxidized and reduced forms. It is important to note that the $\Delta \chi$ values, at the same fields and temperatures, of the MCD spectrum of reduced aconitase are comparable to those of FdII from D. gigas (15). Therefore, the majority of the aconitase sample must contain a reduced 3Fe cluster although the activity is 66% of the maximum value.

In the Mössbauer investigations, dithionite-reduced aconitase exhibited ~5% of maximal activity and 25% of the Fe was in the form of a "$g = 1.94$" EPR signal which is characteristic of a [4Fe-4S]$^{2+}$ cluster (6). In contrast, the dithionite-reduced sample used for MCD investigations exhibited 66% of maximal activity and no EPR signals. We have tried repeatedly to observe "$g = 1.94$" EPR signals for aconitase samples reduced with dithionite alone, without success. However, such signals were observed when extraneous Fe(II) ion was present, as discussed in a later section. The variability in the magnitude of this EPR signal noted by Kent et al. (6) probably relates to differences in the extraneous Fe concentration for individual sample preparations.

There has been little consensus in the literature as to the activity of dithionite-reduced aconitase samples. Estimates range from 0 to 80% of the maximum activity (5, 6, 8–10).

* A. J. Thomson, P. M. A. Gadshy, J. Peterson, A. J. M. Richards, and S. J. George, unpublished observations.
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Fig. 6. MCD spectra of dithionite-reduced aconitase (preparation 1). Anaerobic reduction with a 10-fold stoichiometric excess of dithionite, incubated at room temperature for 10 min. Protein concentration = 65 mg/ml, 50% v/v ethylene glycol. Conditions: temperatures, $1.60$ K (-----), $4.22$ K (---), $18.5$ K (O), $34.0$ K (●); magnetic field = $4.91$ Tesla, pathlength = $1.23$ mm.

Fig. 7. Near IR MCD spectrum of dithionite-reduced aconitase (preparation 1). Conditions as for Fig. 6, temperature = $1.6$ K, magnetic field = $4.91$ Tesla.

Presumably this variability is due to differences in aconitase samples isolated in different laboratories. For preparations from this laboratory, using buffers rigorously free of extraneous Fe, we have consistently been able to obtain up to 70% of maximal activity by anaerobic incubation with dithionite. The MCD results suggest that the Fe-S cluster in this derivative is predominantly a reduced 3Fe center. However, caution must be exercised in attributing catalytic activity to the presence of a reduced 3Fe center. First, it is possible that cluster transformation is induced during the assay procedure, subsequent to spectroscopic sampling. The reduced 3Fe cluster in aconitase has a high affinity for Fe, resulting in the reformation of a [4Fe-4S]$^{2+}$ cluster. Second, a fraction of the Fe may be in the form of diamagnetic [4Fe-4S]$^{2+}$ clusters, since MCD signals of such centers are weaker by a factor of 30 at $1.5$ K and $5$ Tesla. In the latter case, however, both the consistency of the magnetization data for the principal MCD bands and the magnitude of the MCD spectrum, compared with that of reduced $D. gigas$ FdI under analogous conditions (15), argue against any appreciable heterogeneity in the cluster content.

Activity had previously been exclusively linked to the formation of a diamagnetic [4Fe-4S]$^{2+}$ center (6). To ascertain whether a [4Fe-4S]$^{2+}$ cluster could be generated via "cannibalization" of reduced 3Fe centers, a sample of aconitase was incubated with a 20-fold excess of dithionite for 30 min at room temperature. Both the activity and the magnitude of the MCD spectrum were unchanged for this larger excess and
The weak paramagnetic component with broad featureless electron reduction to the catalytically active clusters in Fe(II) ion-treated aconitase. Thus we have no evidence for any dithionite-induced almost maximal activity and no EPR signals assignable to Fe-S centers in aconitase.

Fe(II) Ion-treated Aconitase—Anaerobic incubation of aconitase with a 10-fold stoichiometric excess of Fe(II) ion for 30 min at room temperature produced a derivative with almost maximal activity and no EPR signals assignable to Fe-S centers. In contrast to the dithionite-treated enzyme this EPR-silent derivative is characterized by a very weak MCD spectrum in the region 300–800 nm (see Fig. 9); for example at 1.7 K and 4.91 Tesla the MCD signals have $\Delta \epsilon$ values of less than 10 M$^{-1}$ cm$^{-1}$. This is comparable with $\Delta \epsilon$ values observed under these conditions of field and temperature for diamagnetic Fe-S clusters (26) and is an order of magnitude less than that seen for paramagnetic clusters (26). Above 400 nm the spectrum shows weak temperature dependence. The sharp derivative centered at 420 nm is attributed to a trace of high spin Fe(II) heme impurity (less than 0.1% of total Fe content of sample) (42, 43). The intensity of the spectra at liquid He temperatures is indicative of predominantly diamagnetic clusters in Fe(II) ion-treated aconitase. Indeed at 95 K this diamagnetic species dominates the spectrum since further increase in the temperature results in little spectral change. The form of the MCD spectrum at 95 K is rather featureless and all the bands have a positive sign. This spectrum is not, therefore, a discriminating diagnostic test for cluster-type. However, we note that the MCD spectrum of the [4Fe-4S]$^{2+}$ cluster in reduced Chromatium vinosum high potential iron-sulfur protein (cf. Fig. 3 of Ref. 28) has a similar intensity and resembles the 95 K spectrum in Fig. 9. Therefore, we conclude that the added Fe(II) ion has been utilized to build a [4Fe-4S] cluster from a [3Fe-4S] center. In agreement with the Mössbauer investigations (6), this conversion restores full catalytic competence to aconitase. However, in the activation experiments of Kent et al. (6), activation by addition of Fe(II) ions was carried out with dithionite-reduced protein. For activation of as isolated aconitase it seems likely that excess Fe(II) ion is required for the cluster conversion, since Fe(II) ion is necessary both for incorporation and 1-electron reduction to the catalytically active 2+ redox state. The weak paramagnetic component with broad featureless MCD bands at wavelengths longer than 450 nm may arise from the free Fe(III) ion produced during this process.

Fe(II) Ion Plus Dithionite-treated Aconitase—Anaerobic incubation of aconitase with a 2-fold stoichiometric excess of Fe(II) ion in the presence of a 10-fold stoichiometric excess of dithionite resulted in an EPR-active sample with almost maximal activity. The EPR spectrum, shown in Fig. 10A, consists of a rhombic signal with $g_1 = 2.05$, $g_2 = 1.91$, and $g_3 = 1.84$. EPR signals of this type are characteristic of [4Fe-4S] clusters in the +1 oxidation state. This spectrum is superim-

**Fig. 8.** MCD magnetization plot for dithionite-reduced aconitase. Conditions of measurement as for Fig. 6, wavelength = 710 nm. Temperatures, 1.57 K (+), 4.22 K (x), 10.0 K (O), 30.0 K ( ), magnetic fields between 0 and 4.91 Tesla. Arrow indicates intercept value for the 1.57 K data, $I = 0.18 \pm 0.02 \text{ cm}^2 \text{ mol}^{-1} \text{ K}^{-1}$, Boltzmann constant.

**Fig. 9.** MCD spectra for Fe(II) ion-treated aconitase (preparation 1). Incubated anaerobically with a 10-fold stoichiometric excess of Fe(II) ion for 30 min at room temperature. Protein concentration = 63 mg/ml, 50% v/v ethylene glycol. Conditions: temperatures, 1.70 K (---), 4.22 K (---), 10.0 K (O), 50.0 K ( ), 95.0 K (---); magnetic field = 4.91 Tesla, pathlength = 1.29 mm.
posed on six equally spaced hyperfine lines, shown by arrows below the spectrum, which arise from a Mn impurity in the FeSO₄. Quantitation reveals the rhombic signal to be a minor species accounting for between 0.05 and 0.10 spins/molecule. Fig. 10B shows the corresponding MCD spectra for this derivative at 1.60, 4.22, and 38.0 K and 4.91 Tesla. In accord with the EPR results, the spectrum is comprised of a weak temperature-dependent MCD signal and an underlying temperature-independent spectrum. This is most clearly illustrated by magnetization curves, since they do not saturate at low temperatures and high magnetic fields (not shown). The MCD spectrum of the paramagnetic component is revealed by differencing the 1.6 and 38 K spectra, as seen in Fig. 10C. The EPR and Mössbauer properties of this paramagnet are indicative of a [4Fe-4S]⁺⁺ cluster (5). In the wavelength range 500–800 nm, the MCD spectrum of this species is remarkably similar to that of the [4Fe-4S]⁺⁺ cluster in reduced *Clostridium pasteurianum* Fd (cf. Fig. 4 of Ref. 28) and in *Desulfovibrio africanus* Fd.⁷ Using the EPR spin integration of 5–10% to correct the ΔΔμ scale gives reasonable values of ~50 M⁻¹ cm⁻¹ for a single [4Fe-4S]⁺⁺ cluster. Although the wavelength region 350–500 nm contains peaks in common with the low temperature MCD spectrum of [4Fe-4S]⁺⁺ clusters, there are additional features, notably the trough between 450 and 500 nm, which disrupt the form of the spectrum. We conclude that this aconitase derivative contains a mixture of approximately 90% [4Fe-4S]⁺⁺ and 10% [4Fe-4S]⁺⁺ clusters on the basis of the combined EPR and MCD results.

**CONCLUSIONS**

The results described above further illustrate the potential of low temperature MCD spectroscopy for elucidating the cluster-type and electronic ground state properties of protein-bound iron-sulfur clusters. For aconitase as isolated, MCD spectroscopy provides independent confirmation that the Fe-S cluster has the characteristic electronic properties of a 3Fe center for the oxidized and reduced clusters, respectively. Furthermore, the detailed pattern of electronic transitions revealed in the low temperature MCD spectra establish three classes of [3Fe-4S]⁺⁺ centers in good agreement with resonance Raman data (45, 46) and consistent with the available redox potential data.

The characteristics of aconitase isolated by the rapid procedure (7) make it likely that the native form of the enzyme contains a [4Fe-4S]⁺⁺ cluster. It would appear from this that the [3Fe-4S] cluster in the inactive form may be an artifact of aerobic isolation, although the possibility that such oxidative damage may also occur in the cell cannot be excluded. The process of reconversion of a 3Fe to 4Fe cluster in aconitase is readily monitored via the low temperature MCD spectra and occurs with full recovery of catalytic competency, providing aconitase is exposed to extraneous Fe(II) ion under anaerobic conditions. The surprising result of the present study is that catalytic activity of ~66% of maximal activity is produced by dithionite reduction of the 3Fe center in the absence of added Fe. According to the hypothesis of Kent et al. (6), enzymic activity is associated only with [4Fe-4S]⁺⁺ clusters which are built up from a [3Fe-4S]⁺⁺ core. The iron is supplied as Fe(II) during activation of the enzyme. However, it is possible that additional iron is acquired from adventitious protein-bound iron or from reagents and glassware. One further interesting possibility is that [4Fe-4S]⁺⁺ clusters may build up at the expense of [3Fe-4S]⁺⁺ cores by a process of

⁷ E. C. Hatchikian, R. Cammack, D. Patil, A. J. Thomson, A. J. M. Richards, and S. J. George, unpublished data.
"cannibalization." The oxidized sample of aconitase, preparation 1, contains almost entirely (93%) [3Fe-4S] clusters, as judged by the spin integration of the g = 2.01 signal. The MCD spectrum of dithionite-reduced aconitase, Fig. 6, has an overall intensity similar to that of dithionite-reduced FdII from *D. gigas* (15). Therefore, we conclude that the dithionite-reduced sample contains mainly [3Fe-4S] clusters. Although MCD spectroscopy does not detect directly diamagnetic [4Fe-4S]** clusters in the presence of the overwhelmingly dominating reduced [3Fe-4S] clusters, the magnitude of the signal intensity can be used to assess the amount of reduced [3Fe-4S] cluster. Since the MCD signal intensity in Fig. 6 is that expected for a sample wholly composed of reduced [3Fe-4S] clusters, there can be only a minimal amount of [4Fe-4S]** cluster present. Certainly it does not seem possible to have 66% of the clusters present as diamagnetic [4Fe-4S]** centers as is required by the activity assay and the hypothesis of Kent et al. (6).

One further important observation may be made. We have examined the intensity of the reduced [3Fe-4S] MCD signal as a function of time in the presence of dithionite for a period of 30 min. No change in the MCD signal intensity is observed. Hence there is no "cannibalization" of [3Fe-4S] clusters to rebuild [4Fe-4S]** clusters over this time scale.

We have failed to confirm the finding of Kent et al. (6) that catalytic activity in aconitase is associated only with a cluster of the [4Fe-4S] type. We appear to have significant activity associated with the dithionite-reduced [3Fe-4S] cluster. However, it has not yet proved possible to carry out useful spectroscopy, diagnostic of cluster-type, on the assay mixture, since the assay is performed under conditions of extreme cluster dilution. It is possible that this introduces an ambiguity into the interpretation of all experiments in which a parallel is drawn between spectroscopic assay on concentrated samples and enzymic activity on highly diluted samples. Recent experiments certainly give additional support to the hypothesis that catalytic activity is exclusively associated with the presence of [4Fe-4S] centers. Mössbauer experiments implicate a specific role for the incorporated Fe in relation to the substrate (44). Reductive activation by dithionite in the absence of added Fe(II) ion is prevented by the presence of Fe chelators such as EDTA. Clearly further experimentation is required to unambiguously resolve the question as to whether a reduced 3Fe center alone is capable of promoting any catalytic activity in aconitase.

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REFERENCES

1. Gulsker, J. P. (1968) J. Mol. Biol. 38, 149-162
2. Villafraanca, J. J., and Milbank, A. S. (1972) J. Biol. Chem. 247, 3454-3463
3. Kennedy, M. C., Rausen, R., and Gawron, D. (1972) Biochem. Biophys. Res. Commun. 47, 740-745
4. Emptage, M. H., Kennedy, M. C., and Beinert, H. (1983) Abstracts of the American Society of Biological Chemists Meeting, San Francisco, R. R. Ramsay and T. P. Singer, manuscript in preparation.

*Emptage, M. H., Kennedy, M. C., and Beinert, H. (1983) Abstracts of the American Society of Biological Chemists Meeting, San Francisco, R. R. Ramsay and T. P. Singer, manuscript in preparation.*
Characterization of the Fe-S cluster in aconitase using low temperature magnetic circular dichroism spectroscopy.
M K Johnson, A J Thomson, A J Richards, J Peterson, A E Robinson, R R Ramsay and T P Singer

J. Biol. Chem. 1984, 259:2274-2282.

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