Mössbauer studies of beef heart aconitase: Evidence for facile interconversions of iron–sulfur clusters

(1) EPR/aconitase activation/ferredoxin

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ABSTRACT Beef heart aconitase, isolated under aerobic conditions, has been studied with Mössbauer and EPR spectroscopy. In the oxidized state, the enzyme exhibits an EPR signal at g = 2.01. The Mössbauer data show that this signal is associated with a 3Fe cluster. In dithionite-reduced aconitase, the 3Fe cluster, probably of the [3Fe–3S] type, is in a paramagnetic state of integer electronic spin (S = 2); the Mössbauer spectra exhibit all the unique features reported for proteins with 3Fe clusters. On activation of aconitase with ferrous ion, the paramagnetic 3Fe cluster of dithionite-reduced enzyme is converted into a diamagnetic (S = 0) form. Activation studies with iron enriched in either 57Fe or 59Fe suggest that activation transforms the 3Fe cluster into a center that has a [4Fe–4S] core. This conclusion is supported by the observation that EPR signals characteristic of reduced [4Fe–4S] clusters can be elicited under appropriate conditions. It has frequently been assumed that the activation of aconitase with Fe2+ produces an active site containing a single ferrous ion. The data reported here suggest that a ferrous ion is used to rebuid a [4Fe–4S] cluster.

In the past few years, it has been recognized that many features of Fe–S proteins cannot be understood in terms of the structural types known for these proteins—i.e., FeS4, [2Fe–2S], [4Fe–4S], or [2Fe–4S]. The occurrence of 3Fe clusters has been reported for a few proteins, of which ferredoxin I of Azotobacter vinelandii (1), ferredoxin II of Desulfovibrio gigas (2), and beef heart aconitase (3) are probably the most intensively studied. X-ray diffraction studies on A. vinelandii ferredoxin (Fd) show that these clusters have a [3Fe–3S] core (4). The decisive spectroscopic evidence for the presence of a [3Fe–3S] center has been the observation of an EPR signal at g = 2.01 in oxidized samples together with the unique Mössbauer spectra that these structures yield in the reduced state (5).

Beef heart aconitase has properties not found in other proteins thought to contain [3Fe–3S] clusters. As obtained on routine purification, it is enzymatically inactive, but it can be reactivated by a number of treatments, all of which have in common the reduction of the Fe–S cluster (6, 7). Although inclusion of iron in the activation medium yields the highest activities, iron does not appear to be an obligatory ingredient of such media; activities up to 70% of maximum can be induced by some reducing agents alone (6, 7). We have now studied, by Mössbauer spectroscopy, aconitase samples reduced and activated in a variety of ways. Only samples reduced and activated with dithionite (≤ 30% of maximal activity) have the Mössbauer features characteristic of [3Fe–3S] centers. Those activated with dithiothreitol/Fe2+, dithionite/Fe2+, or dithiothreitol alone dis-

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MATERIALS AND METHODS

Aconitase was purified from beef heart as reported (8) with minor modifications. Analytical methods and EPR spectroscopy were as in ref. 9 and yielded results as in refs. 8 and 7 respectively. Activations were carried out anaerobically; for routine assays (10), 0.1 mM ferrous ethylene diammonium sulfate and 5 mM dithiothreitol were used, and Mössbauer and EPR samples were prepared as desired for the specific purpose. Anaerobic procedures followed the outlines of ref. 11 with appropriate modifications.

Although enzymatic assays for aconitase activity are simple and reproducible, it must be kept in mind that drawing a representative aliquot from a Mössbauer sample (200–700 mg of protein per ml) kept under specific conditions (usually anaerobic) is difficult. Correlations of enzyme activity with features in Mössbauer spectra are therefore not quantitative to the extent eventually desired by us.

Isomer shifts, δFe, are quoted at 4.2 K relative to Fe metal at 298 K.

RESULTS

Dithionite-Reduced Aconitase. Mössbauer spectra of dithionite-reduced beef heart aconitase taken at 4.2 K are shown in Fig. 1. The spectrum of Fig. 1A was recorded in zero applied field and consists mainly (≈75% of total Fe) of two quadrupole doublets, labeled I and II. The quadrupole splittings and isomeric shifts, ΔEo = 1.34 mm/s and δFe = 0.45 mm/s for doublet I and ΔEo = 0.49 mm/s and δFe = 0.30 mm/s for doublet II, are similar to the parameters observed for the reduced [3Fe–3S] clusters of the Fds from D. gigas (2) and A. vinelandii (1). Furthermore, the intensities of the two doublets are compatible with the 2:1 ratio observed for the two Fds. Most important, the two doublets originate from iron sites belonging to a paramagnetic center. This is apparent from the observation of substantial magnetic hyperfine interactions in the presence of a weak applied field, as shown by the broad features seen in

Abbreviation: Fd, ferredoxin.
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The spectrum of Fig. 1B. The drastic change observed in weak applied fields is an unusual feature of reduced [3Fe–4S] clusters (5). Thus, the Mössbauer spectra of dithionite-reduced aconitase exhibit all features characteristic of reduced [3Fe–4S] centers.

In addition to the doublet structure attributed to the reduced [3Fe–4S] cluster, the spectrum in Fig. 1A shows the presence of a broad and featureless component that has a magnetic splitting characteristic of [4Fe–4S] clusters in the +1 oxidation state. Examination of the sample by EPR showed the presence of a species with \( g_1 = 2.06 \), \( g_2 = 1.93 \), and \( g_3 = 1.86 \). After we became aware of the "g = 1.94" type signals in dithionite-reduced aconitase, we examined all our samples with EPR. We observed this species and, depending on the buffer, additional similar species in all preparations, albeit in different concentrations (10–25%). The sample of Fig. 1 had the largest \( g = 1.94 \) component we have observed in a Mössbauer spectrum. One of our preparations gave only the spectrum attributed to the [3Fe–3S] center; there was no evidence for the presence of a \( g = 1.94 \) species (see figure 2 of ref. 3).

After discovering the \( g = 1.94 \) species in reduced aconitase, we searched for conditions that minimized the concentration of these species. Decreasing the pH from 7.5 to 6.5 prevented the formation. Moreover, \( g = 1.94 \) signals were not observed when aconitase was reduced with dithiothreitol. The Mössbauer spectrum of such a sample yielded a surprise. A diamagnetic species rather than a paramagnetic [3Fe–3S] cluster was observed! We present these findings below.

**Oxidized Aconitase.** Beef heart aconitase, as isolated, exhibits an EPR signal centered at \( g = 2.01 \) (7). We will refer to this material as oxidized aconitase. The Mössbauer spectrum of an oxidized sample recorded at 85 K showed a single quadrupole doublet with linewidth 0.3 mm/s (full width at half maximum). The Mössbauer parameters \( \Delta E_Q = 0.71 \) mm/s and \( \delta_\text{Fe} = 0.27 \) mm/s are characteristic of high-spin Fe\(^{3+}\) in a tetrahedral environment of sulfur ligands. The value for \( \delta_\text{Fe} \) is identical to those observed for the Fds from *D. gigas* (2) and *A. vinelandii* (1). The spectra of the latter proteins exhibit somewhat smaller quadrupole splittings; \( \Delta E_Q = 0.63 \) mm/s for the *A. vinelandii* protein and \( \Delta E_Q = 0.54 \) mm/s for the Fd from *D. gigas*. Despite the similarities, these parameters alone do not prove the presence of a [3Fe–3S] cluster in oxidized aconitase since the iron sites of the [4Fe–4S] cluster in oxidized high-potential iron protein from *Chromatium vinosum* (12) have similar parameters.

Mössbauer spectra taken at 4.2 K in applied magnetic fields are shown in Fig. 2. Although the general pattern of the spectrum shown in Fig. 2A differs from that of oxidized high-potential iron protein, the difference is not pronounced enough to rule out the possibility that oxidized aconitase contains a [4Fe–4S] cluster. However, the spectra observed in strong applied fields do provide strong evidence in favor of a [3Fe–3S] cluster.

The Mössbauer spectra of oxidized [3Fe–3S] clusters can be described by the \( S = 1/2 \) Hamiltonian (2)

\[
\hat{H} = \beta S \cdot \mathbf{g} \cdot \mathbf{H} + \sum_j [S \cdot \mathbf{A}_j \cdot \mathbf{I}_j - g_j \beta_\text{H} \mathbf{H} \cdot \mathbf{I}_j + \hat{H}_0(j)],
\]

where \( j \) refers to the three subsites. In Eq. 1, all symbols have their conventional meaning; \( \hat{H}_0 \) describes the electric quadrupole interaction of the \( ^{57}\text{Fe} \) nucleus with its environment. The magnetic hyperfine term in Eq. 1, for the experimental conditions used here, gives rise to an internal magnetic field at the nucleus, \( \mathbf{H}_\text{int} = -A \cdot (S) / g_\text{J} \beta_\text{H} \), where \( (S) \) is the expectation value of \( S \) for a member of the ground doublet. In strong applied magnetic fields, the \( ^{57}\text{Fe} \) nucleus senses an effective field \( \mathbf{H}_\text{eff} = \mathbf{H}_\text{int} + \mathbf{H} \). In an applied field of 6 T, only the \( \langle S \rangle = -1/2 \) state of the \( S = 1/2 \) doublet is appreciably populated at 4.2 K.
After the Mössbauer experiments were completed, the sample was thawed and activated by adding $^{56}$Fe. After an incubation time of 60 min, the activity had increased to 75%, and the sample was frozen. The spectrum of the activated sample (Fig. 3A) shows a single quadrupole doublet; the signature of the reduced [3Fe–3S] center has vanished. The parameters of the doublet, $\Delta E_Q = 1.30$ mm/s and $\delta_d = 0.45$ mm/s, are characteristic of [4Fe–4S] clusters in the +2 state. A similar sample (activated with dithiothreitol/Fe) that exhibited a zero-field spectrum identical to the one that gave the spectrum of Fig. 3A was studied in a 3-T field. The observed spectral pattern showed that the cluster of activated aconitase is diamagnetic. This observation and the quoted values of $\Delta E_Q$ and $\delta_d$ suggest that the [3Fe–3S] cluster has been converted into a structure that has a [4Fe–4S] core. It should be emphasized, however, that the data presented so far do not prove a conversion into a [4Fe–4S] cluster. A structural rearrangement of the [3Fe–3S] center, without change of redox state, giving a system that has a diamagnetic electronic ground state is also compatible with the data of Fig. 3A.

In Fig. 3B we present a spectrum of a sample treated as the $^{56}$Fe-activated sample except that $^{57}$Fe (>90% enrichment) was used for the activation procedure. The spectrum shows the presence of two species. One spectral component, quantitated to >60% of the total $^{57}$Fe, gives a quadrupole doublet that has broad asymmetric absorption lines and values for $\Delta E_Q = 3.2$.

![Diagram](attachment:image.png)

**Fig. 3.** Mössbauer absorption spectra of aconitase at 4.2 K in zero field. (A) Dithionite-reduced enzyme (7.8 mM Fe, natural abundance) activated for 1 hr with 99.9% enriched $^{56}$Fe (2.6 mM). (B) Dithionite-reduced enzyme (1.35 mM Fe) activated for 75 min with 0.3 mM $^{57}$Fe (>90% enriched). About 90% of the intensity of the observed spectrum results from the $^{57}$Fe added for activation. a, Doublet belonging to the fourth subsite of a [4Fe–4S] cluster. (C) Apoconitase incubated for 1 hr with dithionite/$^{57}$Fe.
mm/s and $\delta_{Fe} = 1.25$ mm/s, characteristic of high-spin Fe$^{2+}$. This species is also observed (Fig. 3C) when aconitase is incubated with dithionite/Fe$^{3+}$/; thus, the doublet that has $\Delta E_Q \approx 3.2$ mm/s reflects the activating Hapes/Fe$^{3+}$ system.

Of particular interest is the sharp doublet (labeled a) in the center of the spectrum of Fig. 3B. The values of $\Delta E_Q = 0.80$ mm/s and $\delta_{Fe} = 0.45$ mm/s suggest three possibilities. First, the doublet could reflect a high-spin ferric ($S = 5/2$) ion with fast electronic spin relaxation. Spectra taken in strong applied magnetic fields, however, show that this doublet is associated with a diamagnetic ($S = 0$) complex, thus ruling out a ferric site. The Mössbauer parameters and the observed diamagnetism are compatible with a low-spin ferrous site. We have therefore studied four control samples obtained by incubating, just as with aconitase, $^{57}$Fe/dithionite with Hapes/Tris/bovine serum albumin/apoconitase (Fig. 3C). In none of these experiments did we observe a spectral component such as doublet a. Thus, the formation of the species giving doublet a appears to require the presence of aconitase holoenzyme. This leaves two possibilities: doublet a reflects a subsite of a [4Fe–4S]– cluster or it represents a low-spin ferrous iron site formed only with holaconitase. The latter case is unlikely because no low-spin iron complexes are known for nonheme proteins. Moreover, one would have to postulate the (improbable) formation of a low-spin ferrous complex occurring concomitantly with structural rearrangement of the [3Fe–3S] cluster.

We have mentioned above that decreasing the pH prevented the formation of $g = 1.94$ EPR signals. Preliminary experiments with activated aconitase indicate that, at pH 5, up to 70% of the clusters, as compared with the $g = 2.01$ signal of the oxidized enzyme, can be reduced to give $g = 1.94$ signals. The absence of the $g = 1.94$ species in the spectrum of Fig. 3A could be explained if the pH decreased slightly during the activation procedure.

Finally, we mention the results of some preliminary experiments. Essentially the same observations are made when aconitase is activated in the presence of Fe/dithiothreitol. The major effect of dithiothreitol seems to be more rapid cluster conversion. In activation studies with stoichiometric amounts of Fe, we observed that the activity correlated roughly with the intensity of doublet a. Furthermore, under these conditions, all iron from the activating mixture was readily incorporated into the cluster; no evidence for a Fe$^{3+}$ site, frequently postulated for aconitase action, was found. Experiments in which samples were sequentially activated with $^{57}$Fe and $^{59}$Fe suggest that the iron of doublet a exchanges within a few minutes with that of the activating medium.

**DISCUSSION**

We have presented Mössbauer spectra of aerobically isolated beef heart aconitase. The data demonstrate unambiguously the presence of a [3Fe–3S] cluster in the oxidized and dithionite-reduced enzyme. The conclusions drawn from the Mössbauer data are supported by the observation of an EPR signal centered at $g = 2.01$ in oxidized aconitase. We have also shown that the reduced paramagnetic [3Fe–3S] center is converted into a diamagnetic species when the enzyme is activated under reducing conditions in the presence of iron. The diamagnetic species, characterized by a doublet that has $\Delta E_Q = 1.30$ mm/s and $\delta_{Fe} = 0.45$ mm/s, was observed in experiments in which aconitase was activated with $^{57}$Fe; i.e., this doublet represents iron present in aconitase before activation. When $^{57}$Fe was used for the activation, doublet a, which has $\Delta E_Q = 0.80$ mm/s and $\delta_{Fe} = 0.45$ mm/s, was observed; it also results from a diamagnetic site. Thus, the iron site formed in aconitase on activation is distinguishable from the other three sites. We have argued that doublet a belongs to the fourth subsite of a [4Fe–4S] cluster that is formed by addition of one Fe atom to the [3Fe–3S] cluster. The [4Fe–4S] cluster of activated aconitase thus has two types of iron sites; three iron atoms reside in sites that have $\Delta E_Q = 1.3$ mm/s while a fourth site gives a doublet that has $\Delta E_Q = 0.80$ mm/s. Such clusters—i.e., [4Fe–4S]– cores having a 3:1 site ratio—have been observed in Fds from *A. vinelandii* and *D. gigas* (unpublished results).

Notably, the [4Fe–4S]– cluster of activated aconitase is stabilized in the $+2$ oxidation state. The cluster can, however, be reduced to the $+1$ state, as shown by the appearance of strong $g = 1.94$ EPR signals that can be elicited under certain conditions. These observations further support our claim that a [4Fe–4S]– cluster has been formed on activation.

Oxidation of activated aconitase with either oxygen or Tris(phenanthroline)Co(III)chloride does not give the $+3$ oxidation state of the [4Fe–4S]– cluster. Rather, the [4Fe–4S]– structure seems to convert to a [3Fe–3S]– cluster. This suggestion is supported by the reappearance of the $g = 2.01$ signal and by the Mössbauer spectrum of an $^{57}$Fe-activated sample reoxidized with Co-phenanthroline. The spectrum shows that virtually all $^{57}$Fe (which had been incorporated on activation) was removed from the protein, leaving a structure giving a $g = 2.01$ signal. Thus, the $^{57}$Fe is added to and removed from a specific site. This is also in agreement with the observation that the iron of doublet a exchanges quite rapidly.

It has been reported (14) that iron is not obligatory for activation of aconitase. We have activated a sample with dithiothreitol for a Mössbauer study. The spectrum showed again that a diamagnetic cluster had been formed. We believe that the required iron (and sulfide?) is mainly obtained from cannibalization of some 3Fe clusters. Significantly, only 70% of maximal activity is attained and very long activation times are required. Clearly, however, the iron and sulfur stoichiometries need further attention.

One obvious question arises from this work. Is the conversion to a [3Fe–3S] cluster an attribute of the aconitase mechanism or is this cluster merely an artifact of the aerobic isolation procedure? The possibility of converting a [4Fe–4S] cluster into a 3Fe cluster has been suggested by recent magnetic CD studies of ferricyanide-oxidized 2[4Fe–4S] Fd from *Clostridium pasteurianum* (15). Stimulated by our aconitase results, we embarked on a program to study cluster interconversions in *D. gigas* Fd (unpublished results). We found that the 3Fe center of Fd II can easily be converted into a [4Fe–4S] cluster by incubating the protein with dithiothreitol, Fe, and dithionite. Moreover, we have observed that the [4Fe–4S] cluster can be reconverted into a 3Fe center by treatment with ferricyanide. Finally, our Mössbauer studies showed that a substantial fraction of [4Fe–4S] clusters are transformed into 3Fe clusters when the samples are reduced with dithionite in buffers of high ionic strength. These observations do not answer the questions posed above. They indicate, however, the facility of such cluster interconversions.

This study raises a more general question. Are 3Fe clusters biologically active structures or are they the result of oxidative damage to [4Fe–4S] clusters? It is premature to suggest a definitive answer. It is worth noting, however, that 3Fe clusters can be quite stable in the presence of oxygen. Thus, proteins that have a [4Fe–4S] cluster that is easily converted into a 3Fe cluster can escape irreversible damage from exposure to oxygen. It is tempting to speculate that such cluster transformation might be the root of the oxygen stability of some hydrogenases.

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