Iron–sulfur stoichiometry and structure of iron–sulfur clusters in three-iron proteins: Evidence for [3Fe-4S] clusters

(active aconitase/inactive aconitase/Fe/S²⁻ ratios/extended x-ray absorption fine structure/Fe··Fe distance)

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ABSTRACT Beef heart aconitase contains 3Fe clusters in its inactive and 4Fe clusters in its active form. The fully active form can be restored from the inactive one by insertion of Fe²⁺, whereas S²⁻ is not required. Chemical analyses for iron and labile sulfide yield Fe/S²⁻ ratios of 0.66–0.74 for the inactive and 0.90–1.03 for the active form. Sulfane sulfur (S⁰) was not detected. We propose on the basis of these data that the inactive form may arise from the active one by loss of one iron only per cluster with the sulfur remaining as S²⁻ in a [3Fe-4S] structure. Measurements by extended x-ray absorption fine structure (EXAFS) spectroscopy on the 3Fe form of aconitase yield a Fe··S distance of 2.24 Å and a Fe··Fe distance of 2.71 Å. This Fe··Fe distance is in agreement with that obtained by EXAFS on ferredoxin II of *Desulfovibrio gigas*, another 3Fe protein, but disagrees with Fe··Fe distances observed for the 3Fe cluster of *Azotobacter vinelandii* ferredoxin I by x-ray diffraction—namely, 4.1 Å. We suggest that this difference may be due to the presence of a [3Fe-3S] structure in the *Azotobacter* ferredoxin I crystals vs. a [3Fe-4S] structure in liquid or frozen solutions of aconitase. The [3Fe-3S] cluster has been shown to have a relatively flat twist-boat structure, whereas a [3Fe-4S] cluster could be expected to essentially maintain the compact structure of the [4Fe-4S] cluster. This would explain the differences in Fe··Fe distances. Two possible structural models for a [3Fe-4S] cluster are discussed.

Iron-sulfur (Fe-S) proteins containing three irons per cluster, instead of the usual two or four, have attracted considerable recent interest in this field of research. By spectroscopic techniques (1–7), significant and unambiguous differences have been found between 3Fe and the known 2Fe or 4Fe clusters. A crystal structure has been published for ferredoxin I (Fd I) from *Azotobacter vinelandii* obtained at 2.0-Å resolution (8). It shows that the 3Fe cluster in the tetragonal crystal form of this Fd is an essentially flat six-member ring structure, which can be described as having a twist-boat conformation with distorted tetrahedral coordination around each iron center. One iron atom has a non-cysteine ligand.

According to Mössbauer spectroscopy, all three iron sites in 3Fe clusters are spin-coupled in both the 3+ and 2+ oxidation states (1–3, 9). In the reduced 3Fe cluster, two quadrupole doublets appear with a 2:1 intensity ratio, indicating that there is a pair of equivalent iron atoms and a single iron atom of different characteristics. The most striking difference in the results from x-ray diffraction studies on [2Fe-2S] and [4Fe-4S] vs. 3Fe clusters concerns the Fe··Fe distances. They are found to be 4.1 Å in the 3Fe cluster of Fd I from *Azotobacter* as compared to 2.7 Å in the 2Fe and 4Fe clusters (8).

In a recent communication, the results of studies by extended x-ray absorption fine structure (EXAFS) spectroscopy on Fd II of *Desulfovibrio gigas* have been presented and discussed (10). This protein has a 3Fe cluster and, because it contains a single type of Fe-S cluster per subunit of six kilodaltons, is, among 3Fe proteins, particularly suited for spectroscopy. It has the characteristic Mössbauer and EPR spectroscopic properties of *Azotobacter* Fd I (1, 3). However, EXAFS spectroscopy on *D. gigas* Fd II shows a Fe··Fe distance of 2.7 Å (10). The difference of 1.4 Å between the Fe··Fe distance as determined by x-ray diffraction and by EXAFS spectroscopy is far beyond the limits of systematic errors of these techniques. The authors of the EXAFS study conclude (10) “that either the two 3Fe Fds have substantially different core dimensions, a possibility apparently unique to 3Fe centers among known Fe-S systems in proteins, or that one (or more) of the structural studies is in substantial error.”

The discussion of this startling discrepancy prompts us to report here results on yet another 3Fe protein—namely, beef heart aconitase—which have a bearing on this subject. We present below the results of EXAFS studies on this protein and of analyses for iron and labile sulfide on the active [4Fe-4S] (2) and inactive 3Fe forms of aconitase.

MATERIALS AND METHODS

Aconitase was isolated from beef heart mitochondria by two procedures. The first procedure (A), which is essentially that of Ruzicka and Beinert (11), yielded inactive aconitase of >95% purity, as determined by NaDodSO₄/polyacrylamide gel electrophoresis. The specific activity of this material after anaerobic activation with Fe²⁺ and dithiothreitol was 10–11 units/mg by using the coupled assay system of Rose and O’Connell (12). The second procedure (B) allowed aerobic isolation of aconitase retaining 90% of its native activity. The initial purification steps were the same as referred to above, except that 20 mM Hepes at pH 7.5 was used as a buffer. This allowed affinity elution of active aconitase from CM cellulose with buffer and 0.5 mM *cis*-aconitate, as described by Davies and Scopes (13). This material is *ca.* 80% pure, as determined by electrophoresis as above, and has, without activation, a specific activity of 8–9 units/mg.

Iron was determined after wet ashing as described (14). A semi-micro version of the methylene blue method for labile S²⁻ determination (15) was developed for the range of 1–4 nmol. A millimolar absorptivity of 34.5 mM⁻¹·cm⁻¹ was used based on Na₂S·9H₂O as secondary standard, which, in turn, was stan-

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Abbreviations: Fd, ferredoxin; EXAFS, extended x-ray absorption fine structure; FT, Fourier transform.

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dardized iodometrically against two different lots of $\text{KH}(\text{IO}_3)_2$. This procedure as well as a variant developed for determination of S^{2-} in Fe-S proteins with clusters of unusual stability, such as *D. gigas* Fd II, will be reported elsewhere. Zero-valent sulfur (S^0) in the presence of S^{2-} was determined according to ref. 16; the extraction step was omitted. Recoveries ($\pm$ SEM) of iron added to aconitase samples were $108\% \pm 4\%$ and of S^{2-} were $103\% \pm 3\%$. Table 1 shows the mean $\pm$ SEM obtained with these procedures. All data on aconitase are based on dry weight and a molecular mass of 83 kilodaltons.

The sample used for the EXAFS study was prepared by procedure A and had $27.3 \mu\text{mol}$ of Fe and $38.2 \mu\text{mol}$ of S^{2-} per gram. It was concentrated to 600 mg/ml in a collodion thimble under nitrogen pressure and 0.2 ml was placed in a lucite cell designed for both EXAFS and EPR studies (17). The sample then was frozen in liquid nitrogen and maintained at *ca.* -100°C during data acquisition.

X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory on the wiggler beam line VII-3 (side station) by using $\text{Si}[220]$ monochromator crystals during dedicated running at 3.5 GeV , *ca.* 40 mA , with the 8-pole wiggler field at 13 kG . The data were collected by the fluorescence excitation technique by using an array of $\text{NaI}(\text{Tl})$ scintillation detectors similar to the one used for other studies (18) but modified to fit the VII-3 side station. The data reported are the average of 10 scans comprising *ca.* 6 hr of total acquisition time.

The Fe EXAFS data were energy calibrated by using the internal calibration technique (17) with Fe foil. The raw (averaged) data then were reduced by subtracting a smooth curve fit to an experimentally measured scattering background, subtracting a cubic spline ($7,155$ – $7,725 \text{ eV}$ with spline points at $7,300$ and $7,490 \text{ eV}$), and then normalizing to the atomic falloff modeled by the Victoreen formula (19). The resulting EXAFS data were converted to *k*-space by assuming a threshold energy (E_0) of $7,130 \text{ eV}$ and were used for analysis.

The EXAFS data were analyzed by using phase and amplitude parameters (for Fe-S and Fe-Fe scattering) empirically determined with EXAFS data on the $[\text{4Fe-4S}]$ cluster compound, $[\text{Fe}_4\text{S}_4(\text{SCH}_2\text{CH}_2)_4](\text{Et}_4\text{N})_2$. The details of the curve fitting procedures have been described (20). A listing and figure of the raw (averaged) XAS data are available from R.A.S.

RESULTS

Aconitase, as obtained by purification under aerobic conditions, has to be activated to display its full enzymatic activity (21–24). It has been shown that during the activation process Fe is built into the 3Fe cluster to form a $[\text{4Fe-4S}]^{2+}$ cluster (2). A similar cluster conversion has been demonstrated for Fd II from *D. gigas* (3). For full activation of aconitase, added Fe is required but not S^{2-} .^{||} In previous chemical analyses on purified aconitase, it had been observed consistently that S^{2-} values were somewhat higher than Fe values (11, 23, 25). In view of the uncertainties inherent in the determination of labile sulfide (26), no particular attention had been given to these differences. However, with the elaboration of a sufficiently precise version of the methylene blue method for labile sulfide (see SEM in Table 1), it became apparent that the Fe/S^{2-} ratio in routine

preparations of aconitase, in which the 3Fe form is obtained, was not unity but was between 0.66 and 0.74 in all preparations analyzed (see Table 1). An additional series of eight preparations that had been analyzed by a version of the methylene blue method, which showed a SEM two to three times higher, gave Fe/S^{2-} ratios between 0.65 and 0.80, with an average of 0.73.

A method then was developed for preparation of aconitase of *ca.* 80% purity within 4 hr from the time of extraction. The enzyme contained in this preparation still had 90% of the activity of a fully activated sample and only 10% of its clusters were in the 3Fe form, as determined by EPR. Analyses on these preparations gave Fe/S^{2-} ratios of 0.90 – 1.03 (Table 1). No heme or Fe-S impurity could be detected in these preparations, but it cannot be excluded that some nonchromophoric iron may have been present in the protein impurities. However, we consider this unlikely because the Fe/S^{2-} ratios in all preparations analyzed fell into the range of 0.97 ± 0.07 . This ratio is expected because Mössbauer spectroscopy has shown (2) that activated preparations contain $[\text{4Fe-4S}]$ and not 3Fe clusters and the Mössbauer spectrum of preparations B8–B11 (Table 1) had a $[\text{4Fe-4S}]$ cluster as the major (*ca.* 80%) species.

Odd sulfide-to-iron ratios similar to those observed by us for inactive aconitase had been reported previously for NADH dehydrogenase (28, 29), but it was shown recently by Paech *et al.* (30) that ratios close to unity are found when this enzyme is prepared by a rapid procedure with a minimum of manipulations. These authors propose that in the previous purification procedures (28, 29) iron may have been lost and the corresponding sulfide converted to S^0 and that this S^0 may then, after reduction by endogenous thiol (SH) groups during the methylene blue assay, have been included in the S^{2-} values. However, when the single highly reactive SH group of aconitase was blocked with *N*-ethylmaleimide, there was no change in S^{2-} content. We also have assayed for S^{2-} and S^{2-} plus S^0 (16) in several preparations, in some cases after hours of setting at room

Table 1. Iron and sulfide content of inactive and active aconitase

Aconitase preparation, no.*	Fe/mole [†]	S^{2-} /mole	Fe/S^{2-}
Inactive (A)			
3	2.02 ± 0.08 (4)	3.07 ± 0.05 (4)	0.66
10	2.21 ± 0.14 (4)	3.32 ± 0.22 (4)	0.67
11	2.63 ± 0.09 (3)	3.69 ± 0.11 (3)	0.71
12	2.20 ± 0.04 (3)	2.97 ± 0.09 (4)	0.74
13	2.31 ± 0.07 (3)	3.15 ± 0.08 (4)	0.74
14	2.16 ± 0.05 (4)	3.07 ± 0.07 (4)	0.70
Active (B) [‡]			
1	3.12 ± 0.12 (3)	3.48 ± 0.12 (6)	0.90
2	2.75 ± 0.06 (4)	2.90 ± 0.10 (4)	0.95
5	3.03 ± 0.18 (3)	2.95 ± 0.14 (4)	1.03
6	2.88 ± 0.09 (2)	2.91 ± 0.12 (5)	0.99
8	2.90 ± 0.21 (4)	2.88 ± 0.03 (4)	1.01
9	2.65 ± 0.10 (4)	2.75 ± 0.10 (3)	0.96
10	2.90 ± 0.07 (3)	2.99 ± 0.07 (4)	0.97
11	2.81 ± 0.11 (3)	2.83 ± 0.11 (4)	0.99

Values are given as means $\pm$ SEM; *n* values for individual determinations are shown in parentheses.

* A and B refer to procedures A and B described in *Materials and Methods*.

[†] A molecular mass of 83 kilodaltons is assumed (27). However, it must be noted that not all molecules of the $>95\%$ pure protein contain Fe-S clusters. The numbers given refer to Fe per total molecules not per molecule with an occupied cluster site. According to ref. 2, the latter ratio would be 3 for the inactive and 4 for the active enzyme.

[‡] Fe and S^{2-} contents are calculated on the assumption that the *ca.* 20% protein impurities do not contain significant amounts of Fe and S^{2-} .

^{||} Quantitative analyses for labile S^{2-} in highly purified *D. gigas* Fd II are not available because published methods based on that of ref. 15 are not applicable (J. J. G. Moura and E. Münck, personal communications). Analyses in our laboratory for S^{2-} plus S^0 (see *Materials and Methods*) in two different such preparations of Fd II gave a ratio of 0.66 for $\text{Fe}/(\text{S}^{2-} + \text{S}^0)$, indicating that in this protein also four sulfurs are available for every three Fe to form the $[\text{3Fe-4S}]$ structure under reducing conditions.

temperature, but have failed to find any S^0 . Therefore, the proposal of Paech *et al.* is not likely to apply to aconitase.

The Fe EXAFS data and Fourier transform (FT) for the inactive 3Fe form of aconitase are shown in Fig. 1. Preliminary EXAFS data obtained on two previous preparations showed similar structure although lower signal-to-noise ratio. The results of the best two-term fit to the filtered EXAFS data show that the first FT peak gives an Fe··S distance of 2.24 Å, whereas the second FT peak gives an Fe··Fe distance of 2.71 Å. Inasmuch as the aconitase data were collected at *ca.* -100°C and the model cluster data were collected at room temperature, we have not yet attempted to make a detailed assessment of coordination numbers and Debye-Waller factors. However, the EXAFS and FT shown in Fig. 1 are very similar to the data reported for the 3Fe cluster in *D. gigas* Fd II (10).

DISCUSSION

The cluster that is found to be present by x-ray crystallography in crystals of *A. vinelandii* Fd I has three inorganic sulfur atoms, five cysteines, and an additional non-cysteine ligand. The data presented above show quite clearly that the 3Fe cluster in aconitase has a different structure. We gather together the pieces of evidence that bear upon the nature of the structure of the cluster in aconitase: (i) the EXAFS analysis shows categorically an Fe··Fe distance of 2.7 Å; (ii) the analytical data are consistent with a cluster core structure [3Fe-4S]; (iii) there is no S^0 in the protein; (iv) core extrusion applied to aconitase has yielded largely [2Fe-2S] clusters (27).

These facts lead us to suggest a plausible model (Fig. 2) for the structure of the 3Fe cluster in aconitase. If the cysteine ligand released by removal of an Fe atom rebinds to another Fe remaining in the cluster the structure shown is generated. The S^{2-} content is thereby unchanged. Fe_B is coordinated by two cysteine thiols and two S^{2-} ions (a coordination site similar to that found in [2Fe-2S] clusters). The other two Fe atoms, Fe_A , have coordination sites similar to those of the Fe atoms in a [4Fe-4S] cluster—namely, one cysteine thiol and three S^{2-} ions. The 3Fe cluster in aconitase can be rebuilt into a [4Fe-4S] cluster merely by the addition of Fe^{2+} and a reducing agent. Therefore, the protein pocket that holds the Fe-S cluster contains a minimum of four cysteine ligands.

With the aid of computer graphics we have attempted to con-

FIG. 2. Conversion of a [4Fe-4S] cluster into a [3Fe-4S] cluster involving only loss of an Fe atom (indicated by square box) and retention of four cysteine ligands.

struct a structure from fragments of known Fe-S clusters to see whether reasonable bond angles and distances could be obtained. The [2Fe-2S](S_4)₄ coordination geometry taken from the crystal structure of *Spirulina platensis* Fd (31) was added to an FeS_4 fragment. This resulted in rather long Fe_A-Fe_B distances of *ca.* 3.4–3.6 Å. However, if the bond angles at S_3 and S_4 are compressed to *ca.* 75° to give those typically found in Fe-S clusters the Fe··Fe distance can approach 2.7 Å. To prevent the angle at Fe_B opening up towards 180°, the S atoms S_3 and S_4 can be folded above the plane of the Fe atoms. Thus, a model is obtained with all Fe··Fe distances of *ca.* 2.7 Å, Fe··S distances of 2.3 Å, and Fe-S-Fe angles of *ca.* 75°. This structure also provides a set of three S^{2-} ions favorably positioned on one side of the cluster for rebinding an incoming Fe^{2+} ion for conversion back to a [4Fe-4S] core.

This structure has two noteworthy features. It contains two distinct types of tetrahedral Fe, Fe_A and Fe_B , in the ratio of 2:1, in agreement with the Mössbauer spectrum of reduced 3Fe aconitase which shows two types of Fe (2). The pair of Fe_A would be identified with the pair that receives the added electron in the reduced state. Secondly, the model contains the core structure [2Fe-2S] that has been extruded from aconitase (27).

The only other plausible structure is suggested by the model of Henkel *et al.* (32), in which the [3Fe-4S] core would contain a single μ -3 capping S^{2-} ion and each Fe atom would be bound by only one cysteine ligand. Thus, the core would be bound to the protein by three cysteine ligands only. This model is less likely for the following reasons: all Fe atoms are more nearly equivalent than in the structure given in Fig. 2; an additional cysteine should become free in inactive aconitase, but no dif-

FIG. 1. Fe EXAFS data for inactive (oxidized) aconitase. (a) FT of raw EXAFS data (transform range: $k = 3.5-12.5 \text{ Å}^{-1}$, k^3 weighting); (b) filtered EXAFS by using the filter window shown in a. The main FT peak in a is due to Fe-S scattering and the peak to higher R is due to Fe-Fe scattering.

ferences are observed between active and inactive enzyme in their reaction with sulfhydryl reagents (33). It also should be noted that in the model of ref. 32, all Fe atoms are formally ferrous—i.e., this cluster is more reduced as compared to the oxidized 3Fe cluster of aconitase. Because there is still a lack of appropriate synthetic models of [3Fe-4S] core clusters, no definite conclusions as to the choice between the models can yet be reached. Of interest in this context is a dimeric structure of clusters reported by Christou *et al.* (34), showing the essential features (e.g., $\text{Fe/S}^{2-} < 1$) of the proposed [3Fe-4S] model.

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