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EXAFS studies of the isolated bovine heart Rieske $[2Fe\text{-}2S]^{1+(1+,2+)}$ cluster

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Recently the involvement of one or, more likely, two nitrogen-ligands in the Rieske-type [2Fe-2S] cluster has been reported based on the chemical assay and various spectroscopic analyses, such as EPR, Mössbauer, ENDOR, and resonance Raman, of isolated *Thermus thermophilus* HB-8 protein by Fee and his collaborators. Similarly, the presence of at least one nitrogen ligand was shown in the mitochondrial Rieske [2Fe-2S] cluster. We have conducted EXAFS studies of the Rieske [2Fe-2S] protein isolated from the cytochrome bc_1 complex of bovine heart mitochondria. Standard analysis could not distinguish one or two nitrogen ligands per cluster. However, one nitrogen and three cysteine ligands per cluster was found to be, possibly, a better solution in more comprehensive analysis procedures.

Introduction

The cytochrome bc_1 segment of the mitochondrial respiratory chain contains a binuclear [2Fe-2S] cluster * which has unusual physico-chemical properties, namely, a unique EPR signal ($g_{x,y,z} = 1.80$, 1.90, approx. 2.03) and an atypically high redox midpoint potential (150–330 mV) [1–3].

In the pioneering work, Rieske and his co-investigators [4,5] isolated an iron-sulfur protein from bovine heart mitochondria which was solubilized in the succinylated form retaining the characteristic EPR properties. However, no assay was available to test the functional activity of this protein (generally referred to as the Rieske iron-sulfur protein). More recently, Trumpower and Edwards [6] have isolated a form of the Rieske protein having a molecular mass of 24.5 kDa which is active for reconstitution. They have demonstrated that this protein is required for the reduction of cytochrome c_1, oxidation of ubiquinol, and oxidant induced reduction of cytochrome *b*. Subsequently, improved isolation methods have been reported for the reconstitutively active Rieske iron-sulfur protein [7,8].

Since the discovery of this protein in bovine heart mitochondria, Rieske-like proteins have been found to be widely distributed in a variety of organisms [9–15]. Fee et al. [16] isolated a Rieske-like protein with a molecular mass of 20 kDa from a thermophilic bacterium, *Thermus thermophilus* HB-8. This protein contains four atoms of non-heme iron and four moles of both acid labile sulfur and cysteine per molecule. Chemical analysis, EPR, and Mössbauer data [16] indicated the presence of two identical $[2Fe\text{-}2S]^{1+(1+,2+)}$ clusters; a conclusion consistent with the observation that two electrons are required for complete reduction of the isolated protein and the fact that the EPR signal integrates to a spin concentration of one per two iron atoms. These data indicate that each of the $[2Fe\text{-}2S]^{1+(1+,2+)}$ clusters are coordinated to at most two cysteine residues in this thermophile protein.

Electron nuclear double resonance (ENDOR) studies by Cline et al. [17] on this same thermophile protein showed the presence of resonances in the 11–14 MHz region of the Lamour frequency. This result indicated

* Iron-sulfur cluster structure is expressed usually only with the numbers of iron and acid labile inorganic sulfur in the bracket.

Abbreviations: ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; PMSF, phenylmethylsulfonylfluoride; Im_2FeTPP, bis(imidazole)-$\alpha,\beta,\delta,\gamma$-tetraphenylporphinatoiron (III)Cl

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the presence of at least one strongly coupled nitrogen ligand, possibly a histidine residue, coordinated directly to an iron atom in the Rieske-type [2Fe-2S] cluster, a feature which distinguishes this Rieske cluster from those present in a variety of binuclear $[2Fe\text{-}2S]^{1+(1+,2+)}$ ferredoxins [18]. The lower frequency ENDOR and electron spin echo envelope modulation (ESEEM) analyses also suggested the presence of a second weakly coupled nitrogen signal which may arise from the remote nitrogen of the ligand imidazole ring or a second unidentified species of nitrogen ligand. Resonance Raman studies [19] also indicated the presence of nitrogen ligand(s) in this cluster. Telser et al. [20] subsequently reported that the mitochondrial Rieske [2Fe-2S] cluster is also coordinated to nitrogen(s) and is spectroscopically indistinguishable from that in the protein isolated from the *T. thermophilus* HB-8 cytoplasmic membranes.

The basic structure proposed for the Rieske [2Fe-2S] cluster is one in which the two iron moieties are bridged by two inorganic sulfur ligands and connected to the protein polypeptide chain by four amino acid residues containing nitrogen or sulfur ligands. The precise number of the nitrogen containing ligands, however, has not been established.

In this report, the results obtained from extended X-ray absorption fine structure spectroscopy (EXAFS) studies on the Rieske protein isolated from the bovine heart mitochondrial ubiquinol-cytochrome *c* oxidoreductase (Complex III), are presented.

Methods

Isolation of the Rieske iron-sulfur protein from bovine heart ubiquinol: cytochrome c oxidoreductase

All procedures were performed at 4° C unless otherwise indicated. Complex III was prepared as described in Ref. 8. After addition of antimycin (2 mols per mol cytochrome c_1), the complex was stored for 3–5 days in 200 mM NaCl, 25 mM sodium phosphate buffer (pH 7.2) and 50% glycerol (v/v) at −20° C.

The 600 mg of the complex in 120 ml was diluted with the same volume of a buffer containing 0.02% Triton X-100, 200 mM NaCl, 40 mM sodium phosphate (pH 7.2) and was applied to a 500 ml hydroxyapatite column (see Ref. 21). The iron-sulfur protein and the 6.4 kDa protein were split off from the complex during elution with an 800 ml 0.5% Triton, 250 mM NaCl, 85 mM sodium phosphate, 0.2 mM phenylmethylsulfonylfluoride (PMSF) solution. The 6.4 kDa protein eluted first followed by the iron-sulfur protein. The first 500 ml of the eluate was combined and concentrated 10-fold by ultrafiltration on an Amicon YM 5 membrane. The phosphate concentration was reduced to 5 mM by chromatography on Sephadex G-25 in 0.05% Triton, 200 mM NaCl, 5 mM sodium phosphate, 0.2 mM PMSF. The eluate was applied to a 20 ml hydroxyapatite column, equilibrated with 0.05% Triton, 200 mM NaCl, 10 mM sodium phosphate, 0.2 mM PMSF. The 6.4 kDa protein and the excess of Triton passed through the column. After washing with 10 ml of the equilibration buffer, the iron-sulfur protein was eluted by 0.5% Triton, 350 mM sodium phosphate at room temperature in a volume of 7 ml. Following ultrafiltration on Amicon YM 5 to a volume of 0.7 ml, the protein was mostly in an aggregated state, and was sedimented by centrifugation at $100\,000 \times g$ for 12 h. The sediment in a volume of 0.3 ml was homogenized after addition of 0.3 ml glycerol and stored at −80° C.

Spectroscopic methods and analysis

EPR analysis. The EPR sample was rapidly frozen by immersion of the sample tube into the precooled freezing mixture (1 : 5 mixture of methylcyclohexane/isopentane) at 81 K. Measurements were conducted with a Varian E109 X-band spectrometer interfaced to an IBM PC computer. The sample temperature was controlled by a variable temperature cryostat (Air products and Chemical Inc., LTD-3-110) and was monitored with an Allen-Bradley-type carbon resistor located right below the sample. The magnetic field was calibrated with a proton NMR probe, and on a routine basis using a Varian weak pitch *g*-value standard. The microwave frequency was measured by an EIP model 545 Microwave frequency counter. Ohnishi and Von Jagow [22] have reported that Complex III, extracted and purified from bovine heart mitochondria in the presence of Triton X-100, contains an extremely low concentration of ubiquinone (UQ), about 0.1 equivalent of cytochrome c_1, having an EPR spectrum of $g_{x,y,z} = 1.77$, 1.90, 2.03 in either the ascorbate or dithionite reduced state. As shown in Fig. 1, pure Rieske iron-sulfur protein isolated from this ubiquinol-cytochrome *c* oxidoreductase exhibits an identical EPR spectrum to that of Complex III.

EXAFS analysis. X-rays absorption data were collected at the Stanford Synchrotron Radiation Laboratory during dedicated operation of the SPEAR storage ring [70–30 mA, 3.0 GeV] on beamlines I-5 and II-3 using fluorescence detection [23]. The preparations and model compounds were kept at −100° C or lower in order to minimize the mobility of hydrated electrons and radical intermediates produced during X-ray exposure [24].

The data were analyzed as discussed previously [25,26] and data from both proteins and model compounds were treated identically. The EXAFS modulations after subtraction of the 'isolated atom' contribution, multiplication by k^3 (k is the photoelectron wave vector) and normalization to one iron-absorbing atom is shown in the top of Fig. 2. These data were then Fourier transformed as shown in the bottom of Fig. 2 and the contributions of the respective coordination

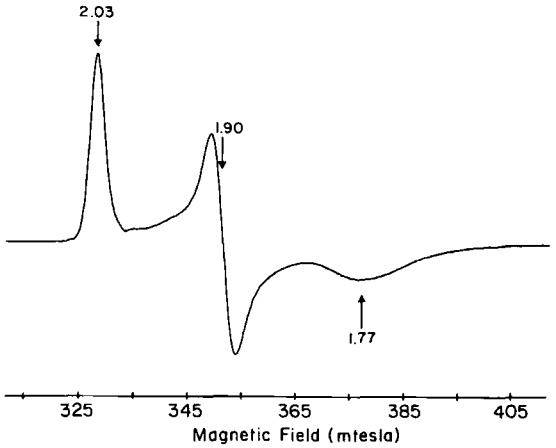

Fig. 1. EPR spectrum of isolated bovine heart Rieske iron-sulfur protein. The protein was isolated from cytochrome bc_1 complex as described in the Methods section. EPR conditions: microwave power, 1 mW; modulation amplitude, $1.25 \cdot 10^{-3}$ tesla; time constant, 0.064 s; microwave frequency, 9.32103 GHz; scanning rate, $5 \cdot 10^{-2}$ tesla/min; spin concentration of the Rieske cluster was 1.5 mM. The protein was reduced with 5 mM dithionite.

shells isolated by Fourier filter and back-transformation.

An estimate of the noise contribution in the filtered data can be obtained by several methods. The data are collected as sample absorbance as a function of energy from which the r.m.s. noise value can be estimated. If the noise is random, data can be simulated having this r.m.s. value noise but no absorption. Alternatively, this estimate can be obtained by subtraction of a smooth function approximating the EXAFS modulations. For either method, the noise spectra are then analyzed by exactly the same procedures as the data from which it was estimated. The back-transformed noise contribution is an estimate of the noise in the filtered data and a sum of the square of these values is a measure of the noise contribution, $\Sigma R^2_{\text{noise}}$, to the sum of residuals squared, ΣR^2.

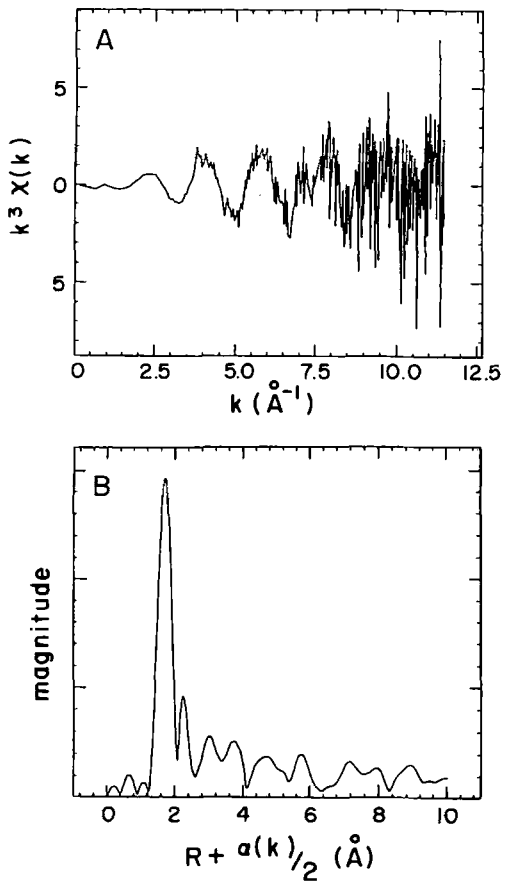

Fig. 2. Iron EXAFS data of reduced Rieske protein. (a) Background subtracted; k^3 multiplied EXAFS data normalized to one Fe atom. (b) Fourier transformation of the above data.

The maximum number of degrees of freedom in the filtered data can be estimated by $\phi d = 2\ \Delta\omega\ \Delta k/\pi$ where Δk is the length of the data used and $\Delta\omega$ is the width of the filter window determined from its area [26,27]. Data from both proteins and models were treated identically. A constrained two atom-type nonlinear least-square fitting procedure was used which describes nitrogen and sulfur ligands: N ligands for each iron having an average distance, r, with Debye-Waller factor, $\Delta\sigma^2$, and threshold, ΔE_0 (Δ = protein − model). The number of ligands per iron was assumed to be four (total of eight for two Fe atoms) and the N parameters were held fixed at values consistent with an integral number of each ligand type. The goodness of fit was judged by the statistical method of ΣR^2, together with the physical reasonableness of the variable values [26–28]. Assuming all the noise in the data is random noise, solutions were judged to be different only if the $\Sigma R^2 > \Sigma R^2_{\text{min}}\ (1 + 1/\nu)$, where ΣR^2_{min} is the sum of residuals of the best physically reasonable solution and $\nu = \phi d - P$, where P is the number of variable parameters in the fit. Total error was conservatively estimated by changing each variable with the others held constant until the ΣR^2 increased by a factor of two on each side of the minimum. Model compounds were chosen carefully to mimic the properties of iron-sulfur proteins: tris-(N,N-dialkyldithiocarbamate)iron (III) (FeCSN) which has six S at 2.44 Å [29] and bis (o-xylyl-α,α'-dithiolato)iron (II) dianion which has four S at 2.36 Å [30]. Bis(imidazole)-$\alpha,\beta,\delta,\gamma$-tetraphenylporphinatoiron (III)Cl (Im_2FeTPP) which has six N at 1.986 Å [31] represents nitrogen ligation. The E_0 values chosen for the nitrogen models differed from those chosen for the sulfur models (ΔE_0 models) by approx. 3 eV.

Results

The data shown in Fig. 2 were analyzed in several different ways using various constraints and assumptions. First, the first shell filtered data (k = 3–11.5 Å^{-1}) was fit using a two-atom-type procedure with the number of each ligand types per Fe atom ($N_{N,S}$) constrained to values that represent integral numbers. These results

TABLE I

Results of two atom-type fitting procedure to the first coordination shell data of reduced Rieske protein with constrained number of ligand values

r in Å; estimated error, ±0.02 Å. N per iron atom values held constant during minimization. $\Delta\sigma^2$ in Å^2, variation ±45% relative to Im_2FeTPP for Fe-N, FeCSN for Fe-S. ΔE_0 in eV; estimated error, ±2.2 eV. $\Delta E_{0\ \text{models}} \approx 3$ eV (see text). * denotes single atom-type fit with N variable.

Fe-N				Fe-S				
r_N	N_N	$\Delta\sigma^2$ ($\times 10^3$)	ΔE_0	r_s	N_s	$\Delta\sigma^2$ ($\times 10^3$)	ΔE_0	ΣR^2
				2.25	4.0	3.1	1.1	23.3 *
2.09	0.5	−5.2	1.3	2.28	3.5	6.6	3.4	1.7
2.10	1.0	−4.3	6.6	2.31	3.0	8.2	4.5	2.3
2.11	1.5	−4.0	9.2	2.33	2.5	7.2	4.6	2.9
2.13	4.0	−1.9	7.7					22.4 *

are given in Table I. It is clear that the better solutions contain N in addition to S ligands. However, the number of each cannot be distinguished.

The idea of 'chemical transferability' of EXAFS phase shifts requires that the ΔE_0 be adjustable [25,27,28,32, 33] and without this, the ultimate precision in determining distances is lost. Hessian matrix analyses [25,27] of the fits of Table I show that the changes in the ΔE_0 value for N ligands within ±10 eV have little effect on the fits and is thus a "don't care parameter" [27]. By choosing a value for the ΔE_0 parameter for Fe-N within this range, this parameter can be held fixed. Subsequently, the number of variables is reduced to five and $1.67 \cdot \Sigma R^2_{min}$ is needed to differentiate solutions. The results of the five variable fits with ΔE_0 for N held fixed at −3.8 eV did not change the ΣR^2 shown in Table I within the accuracy reported. However, solutions with $N_S = 3.5$, $N_N = 0.5$ and with $N_S = 3.0$, $N_N = 1.0$ are distinguished as the better solutions.

In the above methods, Δk is limited to 3–11.5 Å^{-1}. It is a standard procedure to disregard the data from 0–3 Å^{-1} because of the plane wave approximation for the phase and also, the phase and amplitude have been shown to contain multiple scattering effects in this region that are sensitive to chemical environment and geometry [25,28]. However, the ligation of the Rieske protein is dominately sulfur and the model compounds employed here model the physical properties and structure of the iron sulfur proteins very closely [29,30,35,36]. In addition, errors in the plane wave approximation of the phase are also present in the model compounds as both the protein and model data are treated identically. Thus inclusion of these low k data should have little effect on the values of the fitted variables. For $k =$ 0–11.5 Å^{-1} and five variable parameters $1.26 \cdot \Sigma R^2$ is needed to differentiate solutions. The results indicate that, with these assumptions, 3.5 sulfurs at an average distance of 2.27 ± 0.015 Å and 0.5 nitrogen at 2.10 ± 0.01 Å (total of seven sulfurs and one nitrogen for two iron sites) * is the favored solution.

The higher shell peak in the Fourier-transformed EXAFS data (bottom, Fig. 2) at $R + \alpha(k)/2 = 2.25$ Å is similar to that of Fe contribution found for the Rieske-like site of phthalate oxygenase from *Pseudomonas cepecia* [34] and several other iron-sulfur proteins and model compounds [35,36]. Assuming that this peak is due entirely to a similar Fe contribution in the Rieske protein, the Fe–Fe distance is found to be 2.70 ± 0.04 Å.

With this assumption, another approach can also be used to distinguish the first shell solutions. Both shells are filtered as a single contribution. An Fe–Fe contributions similar to that of phthalate oxygenase, other iron-sulfur proteins and of models are subtracted from the data and the resulting first shell contribution is fit with the N values constrained as above. The results of this approach are given in Table II and also suggest that 3.5 S and 0.5 N per Fe atom is the better solution.

Discussion

It is clear that all sulfur or all nitrogen ligation is excluded by all the analysis procedures, regardless of the assumptions or constraints. However, the $(1 + 1/\nu)$ factors used to distinguish solutions are based on the assumption that all the noise in the data is random noise. In this case, solutions which are not overdetermined ($\nu > 0$) having the least number of variable parameters and $\Sigma R^2_{min} \approx \Sigma R^2_{noise}$ are the best solutions. When systematic errors are present, $\Sigma R^2_{min} > \Sigma R^2_{noise}$ and the $(1 + 1/\nu)$ factors for distinguishing fits are conservative, since this factor multiplies the systematic error as well as the noise contributions to ΣR^2_{min}. As $\Sigma R^2_{noise} \approx 0.1$ for the first shell filtered data, the best ΣR^2 values of Table I show that contributions other than random noise are present. Thus the factors used to distinguish these solutions are conservative. Comparison of the residuals for the filtered first shell data with the estimated noise graphically illustrate this in Fig. 3a and the residuals provide information about the nature of the additional contributions. A major contribution arises from the inclusion of a small but non-negligible portion of the higher shell peak by the filter. This situation is unavoidable in filtering these data, since the higher shell is in such close proximity (Fig. 2). This problem, however, is avoided by the last analysis method which filters both shells as a single contribution and subtracts an iron contribution similar to that of phthalate oxidase, other iron-sulfur protein, and models. Since $\Sigma R^2_{noise} \approx 0.24$ for this data, the best ΣR^2 value of

* The two bridging S ligands are counted twice, since they are ligands of both irons.

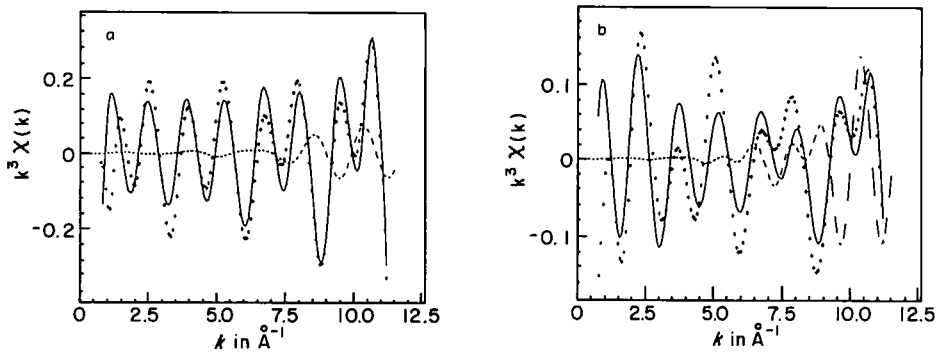

Fig. 3. Comparison of the residuals of the best solutions of Table I and Table II [7S1N (———), 6S2N (+ + +)] to the estimated noise (— — —). (a) The first shell filtered data. (b) The resulting first shell data after filtering of the first and higher shell data as one contribution and subtraction of a higher shell contribution similar to phthalate oxidase, other iron-sulfur proteins, and models.

Table II is approximately that of ΣR^2_{noise} and represents the best possible fit to the data. The residuals of the resulting data are compared to the estimated noise in Fig. 3b. Even though 3.5 S and 0.5 N per Fe, as diagrammed in Fig. 4 is suggested as the best solution by both analysis procedures which involve reasonable estimates and assumptions, the solution of 3.0 S and 1.0 N per Fe cannot be completely excluded.

Recent studies on the amino acid sequence homology of the Rieske iron-sulfur protein from seven different sources, including mitochondria [21,37,38], chloroplast [4] and bacteria [40–43] have revealed a highly conserved polypeptide segment near the C-terminal. This portion is comprised of two fully conserved amino acid sequences, C_{129} T H L G C_{134}------C_{148} P C H_{151} (using amino acid sequence numbers of yeast Rieske protein) containing four cysteine and two histidine residues in the close vicinity. A sequence homology of typical [2Fe-2S] and [4Fe-4S] iron sulfur proteins indicates that the minimum number of amino acid residues between directly liganding residues is two. Since cysteine and histidine are reasonable residues for S and N ligands, the C-terminal side sequence may provide C_{148} and H_{151}. On the other hand, the N-terminal side could provide either C_{129} and C_{134} or H_{131} and C_{134}. Recently, Gatti et al [44] also suggested that the Rieske [2Fe-2S] cluster in the yeast mitochondria is liganded by three cysteine residues (C_{129}, C_{134} and C_{148}) and one histidine (H_{151}).This suggestion was based on the analysis of 11 respiratory deficient mutants, each of which were identified to have a single amino acid replacement in the highly conserved C-terminal region. They obtained evidence indicating that C_{148} is one of the ligands to the cluster. Their data also suggested the involvement of H_{131} in the binding of ubiquinol.

It should be pointed out that the amino acid sequence of the Rieske-type protein of the thermophilic bacteria has, unfortunately, not been determined. Although spectroscopic properties [16,17] of the Rieske-type [2Fe-2S] protein of *T. thermophilus* HB-8 cells are very similar to the Rieske protein in mitochondria, chloroplast, and others [21,37–43], only this thermophile protein contains two identical clusters per molecule while all other proteins contain a single cluster per molecule. Recently, Kutoh and Sone [45] reported the isolation of the cytochrome bc_1 complex from another thermophile, bacillus PS3, which consists of four polypeptides with apparent molecular weights of 29 (cytochrome c_1), 23 (Rieske protein), 21 (cytochrome b) and 14 kDa. This complex is similar to all other Rieske-type proteins having only one [2Fe-2S] cluster per protein molecule, but dissimilar to that isolated from *T. thermo-*

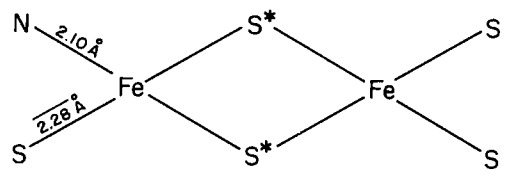

Fig. 4. Schematic diagram of the 1N and 7S solution for the reduced Rieske protein. A bar over the Fe-S distance indicates that it is the average of the seven contributions. Errors are given in the text.

TABLE II

Results of two atom-type fitting procedure to the first coordination shell data of reduced Rieske protein with constrained number of ligand values (higher shell assumed to be Fe-Fe contribution)

r in Å; estimated error, ±0.02 Å. N per iron atom values held constant during minimization. $\Delta\sigma^2$ in Å^2, variation ±45% relative to Im_2FeTPP for Fe-N, FeCSN for Fe-S. ΔE_0 in eV; estimated error, ±2.2 eV. $\Delta E_{0\ models} \approx 3$ eV (see text). * denotes single atom-type fit with N variable.

Fe-N				Fe-S				
r_N	N_N	$\Delta\sigma^2$ ($\times 10^3$)	ΔE_0	r_s	N_s	$\Delta\sigma^2$ ($\times 10^3$)	ΔE_0	ΣR^2
				2.26	4.0	2.7	2.0	21.1 *
2.09	0.5	−8.2	−3.6	2.27	3.5	4.0	2.4	0.51
2.11	1.0	−7.0	5.6	2.27	3.0	4.2	2.8	0.96
2.11	1.5	−5.2	7.3	2.27	2.5	2.7	2.8	1.2
2.13	4.0	−3.5	8.1					55.5 *

philus HB-8 [16]. Therefore it appears that Rieske-type proteins have two different structures (*T. thermophilus* HB-8 versus all other above-cited proteins).

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