Mössbauer Spectroscopy of Non-Heme Iron Proteins*

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ABSTRACT: Mössbauer spectroscopy has been used to study three non-heme iron proteins. The spectra of the ferredoxins from *Chromatium* and from spinach show similar changes on oxidation and reduction. In confirmation of chemical experiments, only one of the two irons in spinach ferredoxin is found to be reduced by sodium dithionite.

The Mössbauer spectra of oxidized high-potential iron protein from *Chromatium* show that the electronic configurations of the four iron atoms are equivalent and are all affected by unpaired electron spins giving rise to broad, diffuse Mössbauer spectra in the temperature range from 60 to 4.6°K. The Mössbauer spectrum of reduced high-potential iron protein is distinctly different from those of the two reduced ferredoxins, and further justifies regarding high-potential iron protein as a non-heme iron protein distinct from the ferredoxin class.

The exact nature of the iron coordination in non-heme proteins is not known, and even basic chemical properties, such as the number of irons functioning in redox reactions, are difficult to understand. It is to this last problem that this paper is addressed.

Mössbauer spectroscopy has been used to study a number of biological materials (Bearden *et al.*, 1965a,b; Lang and Marshall, 1966; Caughey *et al.*, 1966; Gonzer and Grant, 1965). In cases where other information is available, the Mössbauer measurements can often be used to obtain exact information concerning the spin Hamiltonian parameters of the iron ion (Lang and Marshall, 1966; Collins, 1965). The work reported in this paper represents quite a different application; Mössbauer spectroscopy in this instance can be best thought of as a technique for resolving the properties of the iron site from those of the protein as a whole, while keeping the protein intact.

Many reviews exist on the relevance of Mössbauer measurements to chemical parameters (Fluck *et al.*, 1963; Goldanski, 1963, Wertheim, 1964). We will discuss these points only in context; the references will provide any detailed information needed.

Some of the properties of the iron proteins studied here are known. *Chromatium* high-potential iron protein contains four non-heme irons, four labile sulfide atoms, and four cysteine residues per 10,074 formula weight (Dus *et al.*, 1967). The oxidation–reduction potential for this protein is $E_{m,7} = 0.35$ V (Bartsch, 1963). High-potential iron protein shows no ferredoxin activity when tested with the *Clostridium pasteurianum* pyruvate phosphoroclastic assay (private communication from Dr. Howard F. Mower).

Chromatium ferredoxin has been reported by Bachofen and Arnon (1966) to contain three iron atoms and three labile sulfide atoms and possibly five cysteine residues per 6000 molecular weight. However amino acid analyses by Sasaki and Matsubara (1968) indicate that the molecular weight of *Chromatium* ferredoxin is approximately 9800 if six iron and six labile sulfide atoms are assumed. They also report that the protein contains nine cysteine residues. The latter molecular weight is compatible with unpublished observations of Bartsch and Horio who found that *Chromatium* high-potential iron protein and ferredoxin as well as spinach ferredoxin migrate at nearly the same rates through a G-75 Sephadex column, indicating that these three proteins may be of nearly the same molecular size. On the basis of the formula weight derived from amino acid analyses, the ratio of iron : labile sulfide : cysteine in *Chromatium* ferredoxin may be (5 or 6) : (5 or 6) : 9, respectively.

Spinach ferredoxin, with a molecular weight of 10,482 based on the amino acid sequence determination of Matsubara *et al.* (1967), contains two iron atoms and two labile sulfide atoms plus five cysteine residues. Both spinach and *Chromatium* ferredoxins possess oxidation–reduction potentials less than −0.4 V (Fry and San Pietro, 1963; Arnon, 1965; Bachofen and Arnon, 1966).

Experimental Section

The purification of the non-heme iron proteins from *Chromatium* grown in a ^{57}Fe medium has been reported elsewhere (Bearden *et al.*, 1965b). Based on chromato-

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graphic and spectroscopic properties the high-potential iron protein was a homogeneous sample. The *Chromatium* ferredoxin was crystallized and had a purity index of $A_{283\ m\mu}/A_{388\ m\mu}$ 1.39 as compared with the best sample of Bachofen and Arnon which had $A_{283\ m\mu}/A_{388\ m\mu}$ 1.35. These samples were estimated to contain approximately 80 atom % ^{57}Fe.

Spinach ferredoxin was purified by chromatography on DEAE-cellulose and was enriched in ^{57}Fe by application of the iron-exchange technique used with clostridial ferredoxins by Lovenberg *et al.* (1963). The exchange of iron was carried out with 4.2 μmoles of spinach ferredoxin in the presence of sodium mersalyl (obtained from Sigma Chemical Co.), followed by the addition of excess 2-mercaptoethanol. Then the sample was successively desalted by passage through a column of G-25 Sephadex equilibrated with 1 mM Tris buffer (pH 7.8), chromatographed on a column of DEAE-cellulose, again desalted, and finally concentrated by freeze drying. The final sample in the Mössbauer spectrometer cuvet contained 1.2 μmoles of ferredoxin in 0.3 ml of 30 mM Tris buffer (pH 8). If complete equilibration between added ^{57}Fe and protein-bound iron was attained, the sample should have contained 80 atom % excess ^{57}Fe. The purity index of this sample was $A_{260\ m\mu}/A_{420\ m\mu}$ 6. Another sample which had been freed of the impurity absorbing at 260 mμ gave an identical Mössbauer spectrum in the oxidized form; the reduced form was not checked.

High-potential iron protein was in the reduced state as isolated. The oxidized form was prepared by passing reduced high-potential iron protein through a column of Amberlite IR 400 anion-exchange resin (1-cm diameter × 1 cm high) which had been charged with a solution containing 1 mmole of potassium ferricyanide in 5 mM Tris buffer (pH 7.8). The optical spectrum of the material showed that oxidation was complete and that the sample was free of ferricyanide, which remained on the column.

Another sample was oxidized photochemically by illuminating a mixture of the protein and *Chromatium* chromatophores in the presence of air (P. Linck, unpublished results). The chromatophores were removed by centrifuging the reaction mixture at 100,000*g* for 90 min. The high-potential iron protein was then concentrated by precipitation with 90% saturated ammonium sulfate.

The ferredoxins were reduced in a helium atmosphere by adding 10–20-fold M excess sodium dithionite (2–4-fold M excess/iron) dissolved in 100 mM Tris buffer (pH 8) to the sample contained in the Mössbauer spectrometer cuvet which was then sealed under a helium atmosphere.

The standard sample used for the Mössbauer measurements contained 2 μatoms of ^{57}Fe in a total volume no greater than 0.5 ml. To attain the necessary protein concentration several expedients were followed. The spinach ferredoxin samples were lyophilized, then taken up in a small volume of buffer and transferred to the Mössbauer spectrometer cuvet. The *Chromatium* ferredoxin crystals were centrifuged free of ammonium sulfate solution and then dissolved in 50 mM Tris-acetate buffer (pH 7.8). *Chromatium* high-potential iron protein was precipitated with nearly saturated ammonium sulfate, centrifuged, and the supernatant salt solution was discarded. The precipitate was taken up in the Tris-acetate buffer and an appropriate aliquot was transferred to the spectrometer cuvet. Mössbauer spectra were also taken of samples as ammonium sulfate precipitates.

The Mössbauer spectrometer has been previously described (Bearden *et al.*, 1965c). Several modifications have been incorporated. The source is 20 mCi of ^{57}Co in a copper matrix; the constant-acceleration cam has been modified to produce three cycles of spectrometer sweep per rotation of the cam, increasing the duty cycle of the spectrometer from 27 to 81%. The proportional counter gas mixture is 95% Xe–5% N_2, permitting spectrometer operation at higher counting rates without the pulse amplitude deterioration evident in the Xe–CH_4 proportional counters used previously.

Results

The results of the Mössbauer measurements are summarized in Table I. The quadrupole splitting, ΔE, is a measure of the deviation from spherical symmetry of the electron charge cloud about the ^{57}Fe nucleus, and the isomer shift, δE, is a measure of the s electron density at the ^{57}Fe nucleus, as influenced by the shielding of the 3d electrons and covalent sharing of ligand electrons

TABLE I: Mössbauer Parameters of Reduced and Oxidized Iron in Non-Heme Proteins.

Sample	°K	Quadrupole Splitting, ΔE (mm/sec)	Isomer Shift,[a] δE (mm/sec)
Chromatium high-potential iron protein			
Oxidized	77	0.82 ± 0.02	+0.09 ± 0.02
	4.6	Diffuse hyperfine splitting	
Reduced	77	1.18 ± 0.02	+0.12 ± 0.02
	4.6	1.22 ± 0.02	+0.13 ± 0.02
Chromatium ferredoxin			
Oxidized	77	1.20 ± 0.05	+0.19 ± 0.05
		0.48 ± 0.05	+0.19 ± 0.05
	4.6	1.32 ± 0.05	+0.23 ± 0.05
		0.66 ± 0.05	+0.23 ± 0.05
Reduced	77	2.87 ± 0.05	+1.15 ± 0.05
Spinach ferredoxin			
Oxidized	77	0.60 ± 0.02	±0.02 ± 0.02
	4.6	0.61 ± 0.02	+0.02 ± 0.02
Reduced	77	3.18 ± 0.05	+1.17 ± 0.05

[a] Relative to ^{57}Co in copper; iron metal is +0.226 mm/sec relative to copper (Muir *et al.*, 1966).

(Wertheim, 1964). ΔE is measured from the observed spectrum as the magnitude of the splitting of the absorption lines. δE is the energy difference between the position of the center of symmetry of the absorption spectrum and zero absorber velocity relative to a source of ^{57}Co diffused into copper metal.

Figure 1 shows spectra of oxidized high-potential iron protein at three temperatures. At high temperature the electron-spin lattice relaxation time is short enough so that during the measurement time of the Mössbauer interaction, many depolarizations of the electron spin occur. The average internal magnetic field at the ^{57}Fe nucleus is thus zero and only sharp quadrupole lines occur. Upon decreasing the temperature, the electron spin can retain a given orientation for a longer period; the internal magnetic field no longer averages to zero, and the magnetic moment of the ^{57}Fe nucleus interacts with the nonzero magnetic field. This gives rise to a magnetic hyperfine splitting of the absorption lines in addition to the electric quadrupole splitting, and results in broadening of the sharp lines into a diffuse, unresolved pattern (Bradford and Marshall, 1966). This low-temperature broadening is characteristic of iron proteins with unpaired 3d electrons (Lang and Marshall, 1966). Figure 2 shows the spectrum of reduced high-potential iron protein at 4.6°K, with sharp absorption peaks in contrast to the result for the oxidized form of the protein.[1]

Figure 3 shows the spectrum of oxidized *Chromatium* ferredoxin at 77°K. The shoulders on the inside of the absorption peaks indicate that two types of iron are present in the molecule. In the spectrum of the reduced protein (Figure 4) a new, wide pair of absorption lines appears, indicating reduced iron. One member of this pair is centered at +2.5 mm/sec, and the other is superimposed on the line near −0.4 mm/sec, producing the apparent stronger intensity of the left-hand member of the two major lines.

Figure 5 shows the spectra of oxidized and reduced spinach ferredoxin at 4.6°K. The oxidized form shows only a single pair of lines, unlike the *Chromatium* ferredoxin. The reduced form shows a wide-split pair of lines (one member is the line near +2.8 mm/sec and the other is superimposed on the oxidized line at about −0.4 mm/sec) like those seen for the reduced iron in *Chromatium* ferredoxin.

The parameters given in Table I for the two types of iron in oxidized *Chromatium* ferredoxin were obtained by drawing a superposition of two curves which best fit the observed absorption pattern. The accuracy of this procedure was estimated by varying the parameters until they obviously did not fit the data. The parameters

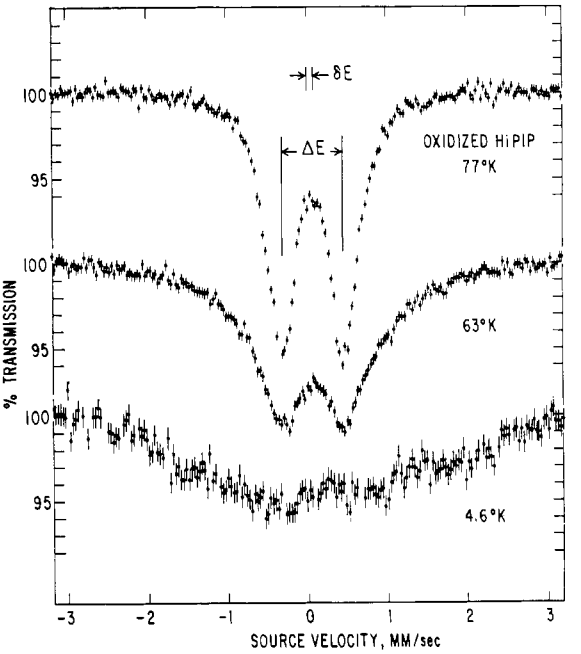

FIGURE 1: Changes in the Mössbauer absorption spectra of *Chromatium* oxidized high-potential iron protein as the temperature is lowered from 77 to 4.6°K.

for the reduced fraction of the iron in the reduced spinach and *Chromatium* ferredoxin were similarly obtained by assuming a superposition of the wide-split (>2.0 mm/sec) pair of lines on the respective oxidized spectra.

Discussion

Comparison of the reduced and oxidized Mössbauer spectra of *Chromatium* high-potential iron protein shows that the electron configuration of all four of the iron atoms are altered on oxidation of the protein. The change in the Mössbauer spectrum on oxidation of high-potential iron protein is most clearly seen at 4.6°K. The spectrum of the oxidized material (Figure 1) is completely broken into diffuse, unresolved hyperfine lines. There is no trace of the sharp lines seen in the spectrum of the reduced protein at 4.6°K (Figure 2). At higher temperatures neither form shows magnetic hyperfine splitting, but the quadrupole splitting of the oxidized line pair is clearly different from the absorption lines of the reduced material. The results are identical for the chemically or photochemically oxidized samples. The Mössbauer data thus indicate that the electronic configurations of the four iron atoms in oxidized high-potential iron protein are identical, but different from those of reduced high-potential iron protein.

The number of oxidation–reduction equivalents of *Chromatium* high-potential iron protein was measured by titrating the freshly oxidized protein with a limiting amount of NADH[2] in the presence of a catalytic quantity of *N*-methylphenazine methosulfate, according to the method of Van Gelder and Slater (1962). In six independent titrations, be-

[1] We reported earlier (Bearden *et al.*, 1965b) that only a small change in the Mössbauer spectrum occurred upon oxidizing high-potential iron protein; this sample was later found to have been entirely rereduced. The present data are for two samples of oxidized high-potential iron protein, oxidized chemically and photochemically as described in the Experimental Section; the two agree completely. Both samples were examined by optical spectroscopy after the Mössbauer experiments and found to be fully oxidized.

[2] Abbreviation is as listed in *Biochemistry* 5, 1445 (1966).

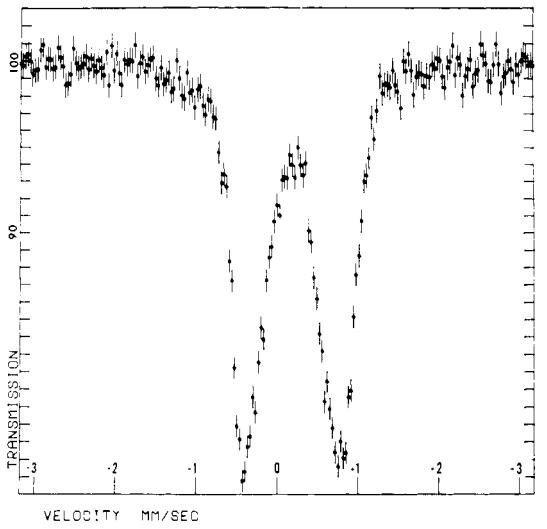

FIGURE 2: Mössbauer absorption spectrum of reduced *Chromatium* high-potential iron protein, 4.6°K.

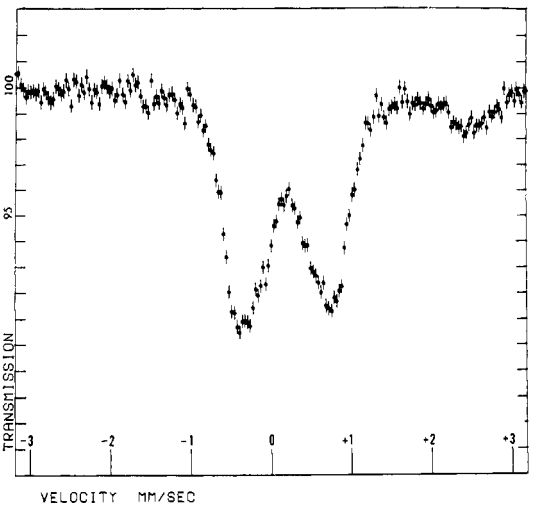

FIGURE 4: Mössbauer absorption spectrum of *Chromatium* ferredoxin reduced with dithionite, 77°K.

tween 1.4 and 1.45 moles of NADH were required to reduce 1 mole of oxidized high-potential iron protein. This evidence indicates that high-potential iron protein may be capable of a three-electron equivalent reaction. The most probable direction of error in the titration experiments is an over estimation of the amount of reductant needed, due to incomplete removal of O_2 from the reaction mixtures despite strenuous efforts to deoxygenate them. It is conceivable, then, that despite the consistency of the titrations, only 2 instead of 3 equiv are involved in the high-potential iron protein oxidation. What is most important is that to make a two- or three-electron equivalent oxidation compatible with the Mössbauer spectra which indicate identical irons it is necessary to assume that the two or three unpaired electrons are shared among the four irons in an unusual delocalized electronic configuration. This conclusion can only be elaborated, however, when independent methods have been developed for verifying the number of electrons involved in the high-potential iron protein redox reaction.

The Mössbauer spectrum of the reduced form of high-potential iron protein has been previously discussed (Bearden *et al.*, 1965b). The quadrupole splitting and isomer shift are fully compatible with diamagnetic iron(II), the configuration indicated by magnetic susceptibility measurements (Ehrenberg and Kamen, 1965). There is a slight asymmetry of the absorption peaks, probably due to the superposition of the more narrowly split absorption of a small amount of oxidized mate-

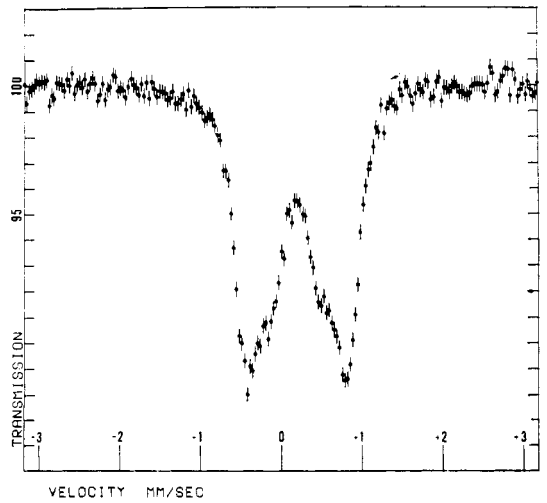

FIGURE 3: Mössbauer absorption spectrum of oxidized *Chromatium* ferredoxin, 77°K.

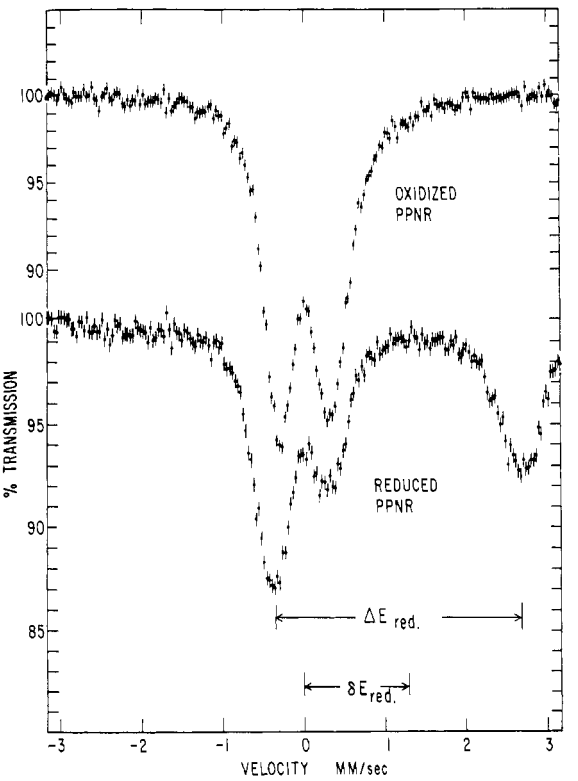

FIGURE 5: Mössbauer absorption spectra of oxidized and reduced spinach ferredoxin, 4.6°K.

rial. This small asymmetry does not allow any hypothesis of two different iron sites per molecule.

Chromatium Ferredoxin. The Mössbauer spectrum of the oxidized form of *Chromatium* ferredoxin shows that there are two types of iron sites in the oxidized protein molecule. The shoulders on the inside of the predominant absorption peaks represent about one-third of the total intensity. If an equal probability of Mössbauer recoil-free absorption (f value) at the sites is assumed, this result indicates that the two types of iron sites are in the ratio 1:2. The assumption of equal probability of Mössbauer absorption is almost certainly valid at 4.6°K, where the modes of atomic vibration which tend to reduce this probability are nearly nonexistent (Kagan, 1961). The fact that the ratio of the inner and outer lines is constant as the temperature is raised to 77°K is also strong evidence that the lattice vibrations have the same frequency spectrum at the two sites, so that the Mössbauer absorption intensities relate directly to chemical stoichiometry.

It is probable that the two lines represent ferric ions in two different sites. However, the possibility that one of the pairs of lines represents ferrous ions inaccessible to oxidizing agents in the native protein cannot be completely ruled out. This possibility appears unlikely, though, because of the observation that the addition of reducing agents causes the appearance of a third species of iron (see below) rather than a simple increase in one of the two forms observed for the oxidized protein.

The Mössbauer spectrum of ferredoxin reduced with dithionite shows that about one-sixth of the total iron is changed to a form characterized by a wide (3.18 mm/sec) quadrupole splitting and a large positive isomer shift, indicative of ferrous iron. Again, relative intensities are the same at 4.6 or 77°K, indicating identical f values for the reduced and oxidized iron. Treatment with a second 10-fold M excess of dithionite did not appreciably increase the fraction of the absorption in the wide-split pair. These data indicate that at most only one iron atom per molecule is reduced by dithionite treatment. The result is consistent with observations Bachofen and Arnon, 1966) that dithionite reduction does not give as complete bleaching of the *Chromatium* ferredoxin chromophore as does the illuminated chloroplast system.

The wide quadrupole splitting and large isomer shift of the reduced fraction of the iron in *Chromatium* ferredoxin are characteristic of high-spin iron(II) (Fluck *et al.*, 1963). This is in contrast to the low-spin iron of reduced high-potential iron protein.

Spinach Ferredoxin. The comparison of the reduced and oxidized spinach ferredoxin spectra shows that only one of the two iron atoms per molecule is susceptible to chemical reduction. The oxidized form displays only a single pair of lines, indicating nearly identical ferric iron environments. On reduction, one-half of the absorption (measured at 4.6 or 77°K) is shifted to a wide-split pair with isomer shift and quadrupole splitting characteristic of high-spin iron(II), just as is seen in reduced *Chromatium* ferredoxin. Similar to the *Chromatium* protein, one member of the wide-split pair is the line near 2.8 mm/sec, and the other is superimposed on the oxidized line at about −0.4 mm/sec. The change of just one-half of the iron on reduction of the protein agrees well with chemical evidence of Fry *et al.* (1963), and the EPR studies of Palmer and Sands (1966), and shows that in the reduced protein the reduced and oxidized iron electronic configurations are distinct. This is in contrast to the observations on oxidized high-potential iron protein.

In summary, as viewed by Mössbauer spectroscopy, the oxidation–reduction characteristics of spinach ferredoxin and *Chromatium* ferredoxin are very similar, and distinct from *Chromatium* high-potential iron protein. The reduced iron in spinach and *Chromatium* ferredoxin is clearly high spin, while the reduced iron in high-potential iron protein is low spin. This distinct difference is further justification for considering high-potential iron protein to be a unique nonheme protein outside the class of ferredoxins, despite the similarities in molecular weight and relative iron, labile sulfide, and cysteine content. Also important, the oxidized and reduced fractions of the iron in the reduced ferredoxins are clearly resolvable as two distinct pairs of quadrupole absorption lines in the Mössbauer spectroscopy. On the other hand, the electronic configurations of the four irons are not resolvable in oxidized high-potential iron protein, so that sharing of electrons between the irons is a distinct possibility.

Acknowledgments

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Studies on the Mitochondrial Adenosine Triphosphatase System. III. Isolation from the Oligomycin-Sensitive Adenosine Triphosphatase Complex of the Factors Which Bind F_1 and Determine Oligomycin Sensitivity of Bound F_1*

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ABSTRACT: The oligomycin-sensitive adenosine triphosphatase (ATPase) complex after extraction with salt solutions in relatively high concentrations (>2 M) is resolvable into a soluble and particulate fraction. The soluble fraction contains protein which can be identified with F_1 subunits. The particulate fraction contains 20% by weight of lipid and is devoid of ATPase activity. When F_1 is added back to the particulate fraction, there is rebinding of F_1 to the particles and this rebinding can be correlated with the restoration of oligomycin-sensitive ATPase activity. The salt-extracted, ATPase-free residue after exposure to dilute alkali (pH 11.5) can still bind F_1 but the particle-bound ATPase thus reconstituted is not oligomycin sensitive. The thesis that there is a specific protein required for binding of F_1 as well as a specific protein for conferring oligomycin sensitivity is compatible with the experiments described in the present communication.

We have previously reported the isolation from bovine heart mitochondria of an oligomycin-sensitive ATPase complex (O-S ATPase)[1] which was nearly free of cytochromes and which was particulate in the absence of bile salts (T agoloff *et al.*, 1968). The enzyme complex, purified fivefold, retained the same sensitivity to inhibitors, the same degree of activation by various divalent metals, and the same specificity toward various triphosphonucleotides as the ATPase of submitochondrial particles. This preparation has proved to be an excellent starting material for studies of the components of the oligomycin-sensitive ATPase.

The separation, from the O-S ATPase, of a soluble, oligomycin-insensitive, cold-labile ATPase identified

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[1] Abbreviations not listed in *Biochemistry 5*, 1445 (1966), are: O-S ATPase, oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff *et al.* (1968); ATPase (NaCl), O-S ATPase extracted with a solution 2 M in NaCl; ATPase (NaCl–urea), O-S ATPase extracted with a solution 2 M in NaCl and 2 M in urea; ATPase (NaBr), O-S ATPase extracted twice with solutions 3.5 M in NaBr; ATPase (NaBr, NH_4OH), ATPase (NaBr) extracted twice with solutions 0.4 M in NH_4OH; F_1, coupling factor I (Pullman *et al.*, 1960); sucrose-Tris, a solution 0.25 M in sucrose and 0.01 M in Tris-acetate (pH 7.5).