Renal Mitochondrial Ferredoxin Active in 25-Hydroxyvitamin D_3 1α-Hydroxylase. Characterization of the Iron–Sulfur Cluster Using Interprotein Cluster Transfer and Electron Paramagnetic Resonance Spectroscopy†

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ABSTRACT: The iron–sulfur protein component of chick renal mitochondrial 25-hydroxyvitamin D_3–1-hydroxylase, purified to apparent homogeneity from vitamin D_3 supplemented animals, has been shown to transfer electrons from NADPH-reduced flavoprotein to cytochrome P-450 in the reconstituted 1α hydroxylation of 25-hydroxyvitamin D_3 [Yoon, P. S., & DeLuca, H. F. (1980) *Biochemistry* (preceding paper in this issue)]. Purified chick renal ferredoxin exhibits low-temperature electron paramagnetic resonance (EPR) properties in the reduced state with prominent signals at $g_z = 2.02$ and $g_y = g_x = 1.94$. We have utilized these paramagnetic spectral characteristics to identify the type of iron–sulfur cluster contained in the chick renal ferredoxin, based on previous observations that other 2Fe2S- and 4Fe4S-containing ferredoxins undergo reversible unfolding, enabling displacement by the thiol carrier, *o*-xylyl-α,α'-dithiol, and interprotein transfer of intact iron–sulfur centers. Chick mitochondrial ferredoxin, when denatured by incubation in dimethyl sulfoxide and renatured in the presence of excess spinach and *Bacillus polymyxa* type I apoferredoxins, as well as carrier thiol ligand, was found to undergo thiol-mediated cluster transfer specifically to the 2Fe2S acceptor, spinach apoferredoxin. Reconstitution of intact iron–sulfur clusters with the apoprotein from spinach occurred in 81% recovery of detectable centers, with <1% transfer of clusters to the 4Fe4S-depleted apoprotein isolated from *Bacillus polymyxa*. The appearance of the $g_z = 2.04$ signal characteristic of reduced spinach ferredoxin, with concurrent loss of resonance at $g_z = 2.02$, indicated that transfer of intact clusters had occurred upon renaturation. These reesults demonstrate that the avian mitochondrial ferredoxin isolated from vitamin D replete animals contains a Fe_2S_2 rather than a Fe_4S_4 type cluster at the active site of electron transfer.

Recent findings on the renal 25-hydroxyvitamin D_3–1α-hydroxylase have demonstrated that this system is a three-component mitochondrial monooxygenase consisting of a flavoprotein, an iron–sulfur protein, and a cytochrome P-450 terminal oxidase which derives reducing equivalents from NADPH for the 1α-hydroxylation of 25-OH-D_3.[1] The oxygen of the C-1 hydroxyl group has been shown to be derived specifically from molecular oxygen, rather than water, characteristic of a mixed-function oxidase mechanism (Ghazarian et al., 1973).

The iron–sulfur component has been purified to homogeneity and was found to have electron paramagnetic resonance properties similar to those of several mammalian mitochondrial ferredoxins found in other steroidogenic tissues, such as the adrenal cortex and testes (Yasunobu & Tanaka, 1973; Orme-Johnson & Sands, 1973).

A method for the determination of the type of cluster contained in iron–sulfur proteins has been developed by Gillum et al. (1977). Bale (1974) found that all four thiol ligands of an iron–sulfur protein could be exchanged for other thiol ligands, leaving the cluster intact. Hence, by adding a large excess of a carrier thiol ligand to an unknown protein solution, the cluster is displaced from the protein, creating a protein-free iron–sulfur cluster. The cluster could then be identified using optical absorption spectrophotometry. This method has been successfully used to characterize a number of different iron–sulfur containing proteins (Erbes et al., 1975; Gillum et al., 1977).

There are, however, several disadvantages to the optical method of cluster identification. The displacement reaction is carried out in 80% of an organic solvent such as dimethyl sulfoxide. This high concentration of solvent causes protein denaturation (Hill et al., 1977), exposing the cluster to solvent, thereby enabling the displacement reaction to proceed. However, if the protein is insoluble in this solvent system, samples are turbid, reducing the sensitivity of this method of detection. In addition, the presence of other chromophores such as heme and flavin moieties complicates the optical absorption spectra. Thus, alternative methods for iron–sulfur cluster identification have been developed (Orme-Johnson & Holm, 1978; Gillum et al., 1977; Kurtz et al., 1978; Coles et al., 1979; Bale and Orme-Johnson, unpublished experiments) including use of electron paramagnetic resonance spectrometry, which clearly separates those resonances due to reduced iron–sulfur centers from those of contaminating coenzyme moieties. Difficulties due to turbidity of the treated samples containing insoluble cluster-depleted apoproteins are avoided when they are examined by EPR in the frozen state at low temperatures.

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, (24*R*)-24,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; DTT, 1,4-dithiothreitol; KP_i, potassium phosphate buffer; Me_2SO, dimethyl sulfoxide; EPR, electron paramagnetic resonance.

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Biochemical and physiochemical characterization of purified chick renal mitochondrial ferredoxin is described in detail in the preceding paper (Yoon & DeLuca, 1980). In the present investigation, thiol-carrier mediated transfer of intact iron–sulfur centers from native chick renal ferredoxin to acceptor apoproteins prepared from 2Fe2S and 4Fe4S ferredoxins,[2] as detected by low-temperature EPR, has been utilized to characterize the type of iron–sulfur center contained in the chick mitochondrial ferredoxin of the 25-hydroxyvitamin D_3-1α-hydroxylase.

Materials and Methods

Cytochrome *c* (type III), NADPH, 2-amino-2-hydroxymethyl-1,3-propanediol and its hydrochloride salt (Tris), and methyl viologen were obtained from Sigma Chemical Co (St. Louis, MO); DEAE-cellulose microgranular No. 52 was from Whatman Ltd. (Clifton, NJ); Sephadex was from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ); sodium dithionite was from BDH Laboratories, Gallard-Schlesinger Mfg. (Carle Place, NY); dimethyl sulfoxide was from Pierce Chemical Co. (Rockford, IL). *o*-Xylyl-α,α'-dithiol was synthesized according to the method described by Kurtz et al. (1978).

Preparation of Chick Renal Mitochondrial Ferredoxin. Eight-week-old Rock-Cornish Leghorn cockerel chicks maintained on a vitamin D replete, 1.0% calcium diet were obtained from A-D Cooperative Broiler Plant (Arcadia, WI). For interprotein cluster transfer studies, chick renal mitochondrial ferredoxin was prepared from kidney tissue obtained from these animals according to the procedure described previously (Yoon & DeLuca, 1980) with the following modifications. The supernatant fraction from the high-speed centrifugation of osmotically shocked mitochondrial sonic extracts has previously been shown to contain the soluble components which constitute the cytochrome P-450 reductase (Pedersen et al., 1976; Yoon and DeLuca, unpublished experiments). This supernatant fraction was loaded onto a DEAE-cellulose column (3.5 × 17 cm) equilibrated with 50 mM Tris-HCl pH 8.0, at a flow rate of 18 mL/h. The resin was washed with 300 mL of low salt buffer containing 0.18 M KCl and 10 mM Tris-HCl, pH 7.5, at 12 mL/h. The ferredoxin component, as detected by its characteristic adrenocortical flavoprotein dependent NADPH–cytochrome *c* reductase activity, was eluted by gradient elution between 0.18 M KCl and 0.45 M KCl in 10 mM Tris-HCl, pH 7.5. Fractions containing greater than 85.5 enzyme units were pooled and lyophilized to dryness at −50 °C using a Unitrap Model 10-100 lyophilizer.

The dried powder was then dissolved in 15.0 mL of distilled water and loaded onto a Sephadex G-100 (2.4 × 84 cm) column equilibrated with 0.1 M KCl and 50 mM Tris-HCl, pH 7.5. Fifty 7.0-mL fractions were collected at a flow rate of 16 mL/h and assayed for the presence of renal ferredoxin. Fractions containing greater than 42.0 enzyme units were pooled, shell-frozen, and again lyophilized. The residue was then dissolved in 6.0 mL of chilled distilled water and dialyzed overnight against 2 L of 10 mM Tris-HCl, pH 7.5. This dialyzed sample contained renal ferredoxin with a specific activity of 135.2 enzyme units per mg of protein at a concentration of 3.45 μM based upon quantitative evaluation of the EPR spectrum of the dithionite-reduced form. The final concentration of renal ferredoxin was estimated to be 0.18 mM in 0.4 M Tris-HCl, pH 7.5, assuming 76% recovery of EPR-detectable iron–sulfur centers during lyophilization. The concentrated sample was frozen and was stored in liquid nitrogen until use for the iron–sulfur cluster transfer experiments.

Preparation of Bovine Adrenocortical Ferredoxin Reductase. Adrenodoxin[3] reductase was prepared from the "S_2" fraction of Omura & Sato (1967) using fresh adrenocortical scrapings. The flavoprotein was further purified according to the method of Sugiyama & Yamano (1975) using chromatography on an adrenodoxin–Sepharose column. Following fractionation with ammonium sulfate between 30 and 70% saturation and gel filtration on a Sephadex G-50 (1.8 × 59 cm) column, fractions with OD_{272nm}/OD_{450nm} ratios from 7.8 to 6.9 were pooled and frozen at liquid nitrogen temperatures.

Purification of Spinach and B. polymyxa Type I Ferredoxins. Spinach ferredoxin was purified according to the procedure described by Orme-Johnson & Beinert (1969). The purity of the final sample was determined by measuring the ratio of absorbances at 422 and 278 nm, which was found to be $OD_{422nm}/OD_{278nm} \geq 0.4$. The concentration of the spinach ferredoxin was determined spectrophotometrically from the amount of absorbance at 422 nm using a molar absorptivity of $\epsilon(M^{-1}\ cm^{-1}) = 10.4 \times 10^4$ (Orme-Johnson & Beinert, 1969).

The type I ferredoxin from *B. polymyxa* was prepared by the method of Stombaugh et al. (1973). Optical absorbance ratios of the final preparations were $OD_{395nm}/OD_{280nm} \geq 0.55$, and the concentrations were determined spectrophotometrically using a molar absorptivity at 395 nm of $\epsilon(M^{-1}\ cm^{-1}) = 1.6 \times 10^4$ (Stombaugh et al., 1973).

Preparation of the apoproteins from both the purified 2Fe2S (spinach) and the 4Fe4S (*B. polymyxa*, type I) ferredoxins was carried out according to the procedure described by Coles et al. (1979), and the apoproteins were checked for cluster depletion using EPR spectroscopy.

Assay for Chick Renal Ferredoxin. In addition to its ability to reduce the endogenous electron acceptor, cytochrome P-450, chick renal ferredoxin will also reduce artificial electron acceptors such as cytochrome *c* in the presence of NADPH-reduced flavoprotein. The presence of the ferredoxin was therefore detected by reconstituted NADPH–cytochrome *c* reductase activity, which was dependent upon the presence of the purified adrenodoxin reductase. Components of the standard reaction mixture are described in the preceding paper (Yoon & DeLuca, 1980).

Thiol-Mediated Iron–Sulfur Cluster Transfer. Under the following incubation conditions, the free iron–sulfur clusters bound to the thiol ligand are scavenged from solution by added purified acceptor apoproteins. Native chick renal ferredoxin was unfolded by denaturation in 80% Me_2SO, and the cluster was displaced from the protein by added thiol. Two and four iron–sulfur apoproteins were then added in excess, lowering the Me_2SO concentration to 40% by dilution with the aqueous buffer containing the apoproteins, thereby allowing protein refolding and reconstitution of the displaced centers with the appropriate apoprotein acceptor. The newly reconstituted holoferredoxin, with EPR features different from those of the native chick protein, was then reduced with sodium dithionite and quantitated by comparison of signal height of the feature at $g_z = 2.04$ with standards calibrated spectrophotometrically.

The methodology used for these experiments has been described by Orme-Johnson & Holm (1978). The displacement reactions were performed under strictly anaerobic conditions in a side chamber attached to a quartz EPR tube made gas-

[2] "2Fe2S and 4Fe4S proteins" represent those ferrefoxins containing two iron and two acid-labile sulfur atoms, or four iron and four sulfur atoms, per cluster. "Fe_2S_2 and Fe_4S_4 cores" indicate covalently bound iron–sulfur clusters.

[3] The term "adrenodoxin" will be used to denote bovine adrenocortical mitochondrial ferredoxin.

tight with tandem septum seals. Fifty microliters of concentrated 0.176 mM chick renal ferredoxin prepared in 0.4 M Tris-HCl, pH 7.5, was added to 200 μL of a 15 mM solution of *o*-oxylyldithiol in 80% Me_2SO. The mixture was incubated at 25 °C for 30 min to allow unfolding of the native chick protein and displacement of clusters by the thiol ligand. Two hundred fifty microliters of 0.1 M Tris-HCl, pH 8.0, containing an eightfold excess of both spinach and *B. polymyxa* apoferredoxins was then added. After 60 min at room temperature, the samples were reduced with 1 M sodium dithionite and 10^{-4} M methyl viologen, and the contents were shaken from a side chamber into the bottom of an EPR tube, which was then quick-frozen in an isopentane bath immersed in liquid nitrogen. Deoxygenated samples and protein components were added to the chamber using gas-tight syringes. All manipulations of proteins, reagents, and apparatus were carried out under an atmosphere of nitrogen made oxygen-free by passing the gas over a heated copper catalyst.

Frozen samples were then subjected to EPR spectral analysis at 13 K and quantitated by measuring the height of the signal at $g_z = 2.04$ or $g_z = 2.05$ for 2Fe2S or 4Fe4S type clusters, respectively. Signal height was calibrated using the spectrophotometrically determined, reduced purified ferredoxins as standards in buffers of the same composition.

Analytical Methods. Protein concentration was determined by the method of Lowry et al. (1951) or by the microbiuret reaction of Gornall et al. (1949), using bovine serum albumin as a standard.

Electron paramagnetic resonance spectroscopy was performed at 13 K using a modified Varian E-9 spectrometer with 100-Hz modulation frequency (Orme-Johnson & Sands, 1973).

The concentration of the chick renal mitochondrial ferredoxin was determined by comparison of the value of the double integral of the EPR spectrum of the dithionite reduced form with the value of the double integral of a Cu^{2+}·EDTA standard of known concentration.

Results and Discussion

Different apoproteins prepared from both 4Fe4S and 2Fe2S ferredoxins have been found to have different affinities for free thiol–cluster complexes (Bale, 1974; Bale & Orme-Johnson, unpublished experiments). Hence, apoadrenodoxin has been shown to preferentially sequester Fe_2S_2 cores derived from adrenodoxin even in the presence of equimolar concentrations of apospinach ferredoxin. However, by increasing the amount of the apoprotein from spinach, the equilibrium then favored reconstitution of the holospinach ferredoxin, rather than the adrenal iron–sulfur protein. Under the conditions used in the present investigation, an eightfold excess of apospinach acceptor protein resulted in 96% recovery of free clusters displaced from the adrenocortical ferredoxin as intact spinach ferredoxin, based upon detection and quantitation using EPR spectroscopy. Reversibility of protein unfolding of native 2Fe2S and 4Fe4S ferredoxins in solution was found to be highly sensitive to the concentration of the organic solvent in solution as determined by spectrophotometric and EPR characteristics of the renatured holoproteins (Bale, 1974). Following denaturation, the efficacy of the apoproteins was tested with synthetic cluster ligands for their ability to donate iron–sulfur centers to acceptor proteins for the formation of newly reconstituted ferredoxins.

Shown in Figure 1 are EPR spectra of the native holospinach (A) and *B. polymyxa* holoferredoxin I (B) utilized in the present study. After incubation at room temperature in 80% Me_2SO, denaturation occurred to completion within 30 min as detected by the disappearance of characteristic EPR features (Figure 1C). The purified 2Fe2S-containing spinach ferredoxin and 4Fe4S *B. polymyxa* I proteins were, therefore, found to be depleted of their iron–sulfur centers to form purified acceptor apoproteins. The *o*-xylyl cluster complexes have previously been shown not to have paramagnetic spin character under these conditions.

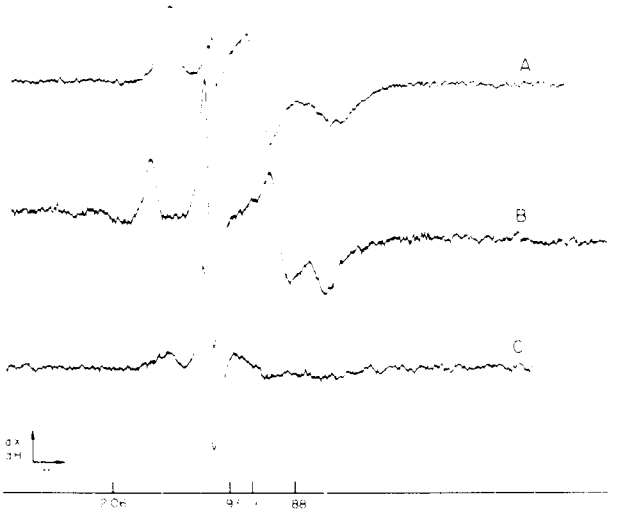

FIGURE 1: Electron paramagnetic resonance (EPR) spectra between 2400 and 4400 G at a microwave frequency of 9.044 GHz of: (a) holospinach ferredoxin; (b) *B. polymyxa*, type I holoferredoxin; and (c) cluster-depleted apoferredoxins. The conditions for EPR spectroscopy were: microwave frequency, 9.05 GHz; modulation frequency, 100 kHz; modulation amplitude, 10 G; temperature, 13 K for all scans, with the following variations. (a) Microwave power, 30 μW; receiver gain setting, 6300; time constant, 1 s; and scanning rate, 250 G per min. (b) Microwave power, 0.3 mW; receiver gain, 1000; time constant, 1 s; and scanning rate, 500 G per min. (c) Microwave power, 30 μW; receiver gain, 10000; time constant, 3 s; and scanning rate, 250 G per min. Field positions are shown on the frequency-independent *g*-factor scale.

Shown in Figure 2 are EPR spectra of chick renal ferredoxin (A) and bovine adrenocortical ferredoxin (B) in the reduced state. The low concentration of the chick iron–sulfur protein, from 24.2 to 25.0 pmol per mg of mitochondrial protein, necessitated the use of crude preparations which were found to contain contaminating flavin and heme chromophores. In addition, intact renal ferredoxin was found to be sensitive to denaturation during lyophilization for time intervals longer than 4 to 6 h at −50 °C, resulting in significant loss of both enzymatic activity and paramagnetic properties upon further purification. The renal ferredoxin was prepared in low buffer concentrations prior to lyophilization to minimize precipitation during subsequent resuspension of the dried residue due to high salt concentrations. Thus, solutions containing the native renal ferredoxin required maximum buffering capacity before freeze-drying, to minimize possible precipitation of protein due to high salt after lyophilization. The concentration of the chick iron–sulfur protein was 3.53 μM prior to a 50-fold increase in concentration necessary for the cluster transfer studies. Despite these problems, the EPR spectrum of reduced chick iron–sulfur protein (Figure 2A) is essentially identical with that of two known hydroxylase ferredoxins, bovine adrenocortical mitochondrial ferredoxin (Figure 2B) and the iron–sulfur protein isolated from *Pseudomona putida* (Orme-Johnson & Sands, 1973). These ferredoxins all show axial EPR spectral properties with narrow resonances and $g_{av} < 2.00$.

Figure 3 illustrates the final reconstituted holospinach ferredoxin obtained from incubating free thiol–cluster com-

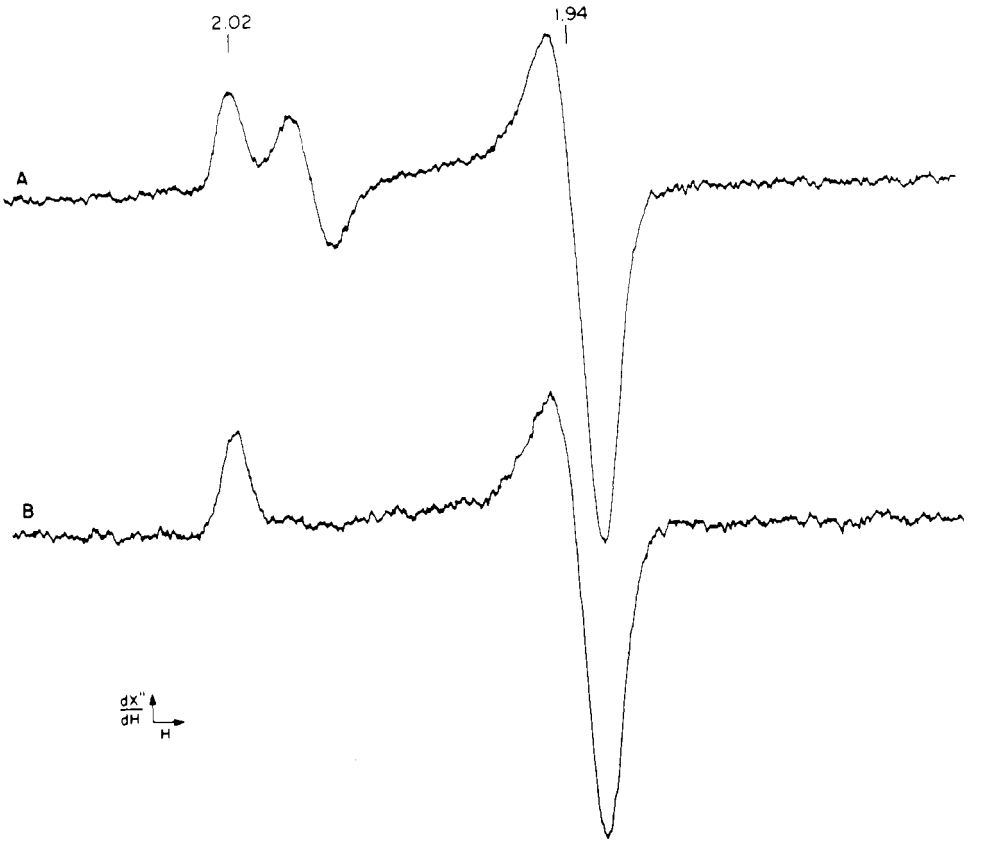

FIGURE 2: Electron paramagnetic resonance spectra between 3100 and 3500 G at a microwave frequency of 9.05 GHz of chick kidney mitochondrial ferredoxin from vitamin D replete animals, as detected by reconstituted NADPH–cytochrome *c* reductase activity, and beef adrenal ferredoxin. (A) Chick renal ferrodoxin in 0.2 M KCl–0.05 M Tris-Cl, pH 7.5, was reduced with sodium dithionite in the presence of methyl viologen. The concentration was determined by double integration of the EPR spectrum and comparison to a Cu^{2+}·EDTA standard of known concentration. (B) Beef adrenal ferredoxin was dissolved in 0.4 M NaCl–0.01 M Tris-Cl, pH 7.4, to a concentration of 9.4 mM based upon the molar absorptivity of the oxidized form at 415 nm of 10×10^4 cm^{-1} M^{-1} (Orme-Johnson and Beinert, 1969) and reduced as in (A). The conditions of EPR spectroscopy were: microwave frequency, 9.05 GHz; microwave power, 10 μW; modulation frequency, 100 kHz; modulation amplitude, 10 G; temperature, 13 K; time constant, 1 s; and scanning rate, 100 G per min. Receiver gain settings for (A) and (B) were 12 500 and 16 000, respectively.

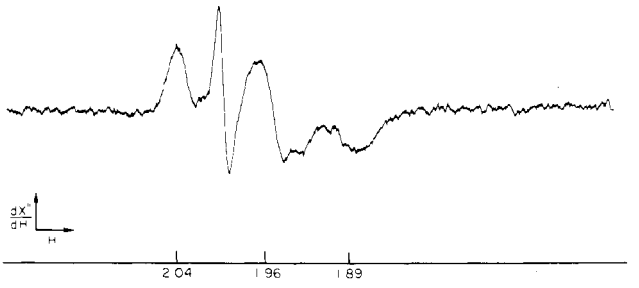

FIGURE 3: EPR spectrum of reconstituted spinach ferredoxin containing Fe_2S_2 type clusters from chick renal iron–sulfur protein following thiol-mediated cluster transfer. The spectrum is from 2400 to 4400 G with conditions for EPR spectroscopy the same as indicated in the legend to Figure 1. Microwave frequency was 9.046 GHz; microwave power, 30 μW; time constant, 1 s; scanning rate, 250 G per min; and receiver gain setting, 16 000. Field positions are also shown on the frequency-independent *g*-factor sacle, and the concentration was found to be 0.112 mM, ad determined by comparison of signal height of the feature at $g_z = 2.04$ with spectrophotometrically calibrated standards.

plexes obtained from the chick protein with both the 2Fe2S and the 4Fe4S acceptor apoproteins. The appearance of the EPR feature at $g_z = 2.04$ characteristic of intact spinach ferredoxin was accompanied by loss of the $g_z = 2.02$ signal of chick renal ferredoxin. Less than 1% of *B. polymyxa* holoferredoxin I was detected, based upon the amount of resonance detected at $g_z = 2.05$.

While previous studies have shown that 4Fe4S proteins transfer 80–100% of intact clusters to *B. polymyxa* apoferredoxin acceptor, and 2Fe2S proteins donate clusters to apoadrenodoxin, no evidence for the transfer of Fe_2S_2 cores to a 4Fe4S apoprotein has been reported. In contrast, up to 20% of Fe_4S_4 complexes may appear as reconstituted 2Fe2S holoferredoxin under some incubation conditions. As can be seen in Figure 3, no reconstituted 4Fe4S–holoprotein could be detected using chick renal ferredoxin as Fe_2S_2 donor, indicating that the concentration of possible contaminating Fe_4S_4 clusters is negligible in these preparations.

The electron paramagnetic spin properties of the iron–sulfur center contained in the chick renal mitochondrial ferredoxin, as well as its ability to undergo thiol-mediated cluster transfer specifically to apospinach ferredoxin, has clearly demonstrated that this component of the 25-hydroxyvitamin D_3–1α-hydroxylase contains a Fe_2S_2 rather than a Fe_4S_4 type cluster at the active site of electron transfer. These results are consistent with the concept that the renal iron–sulfur protein may be classified as a hydroxylase ferredoxin.

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Interaction of Creatine Kinase Isoenzymes with Beef Heart Mitochondrial Membrane: A Model for Association of Mitochondrial and Cytoplasmic Isoenzymes with Inner Membrane†

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ABSTRACT: A beef heart mitochondrial preparation characterized by a high degree of structural integrity, the absence of contaminating myofibrils, and a constant, relatively high level of creatine kinase was employed to study the location and quantitative distribution of the creatine kinase isoenzymes in various subfractions. Submitochondrial fractions studied were (I) the outer membrane–intermembrane space, (II) the total mitoplast, (III) the extrinsic surface (cytoplasmic side) of the inner membrane, and (IV) the inner membrane and matrix space. Determination of the isoenzymes in the fractions by electrophoresis in 0.025 M Tris–0.190 M glycine at pH 8.6 indicated that all fractions except I contained varying amounts of the cytoplasmic muscle-type isoenzyme (MM) and the cationic mitochondrial isoenzyme (MT), the highest proportion of MT creatine kinase being in fraction IV. Comparison of the detergent extracts of whole mitochondria with fraction IV indicated that the MT/MM ratio in the former is ~1:3 and in the latter is ~1:1. DEAE-cellulose chromatography of MM and MT mixtures suggests that the two forms of the enzyme can exist as a complex in solution. The free access of the membrane-impermeable phosphorylcreatine to the enzyme in the everted sonic particles as well as the submitochondrial partition of the enzyme supports the conclusion that the MT creatine kinase is very strongly associated with and probably traverses the inner membrane. A model for the association of beef heart creatine kinase isoenzymes with the inner membrane has been proposed by taking into account our results with those of Addink et al. [Addink, A. D. F., Boer, P., Akabayashi, T., & Green, D. E. (1972) *Eur. J. Biochem. 29*, 47–59] and the properties of the purified MT [Jacobs, H. K., & Graham, M. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1574; Hall, N., Addis, P., & Deluca, M. (1979) *Biochemistry 18*, 1745–1751].

It is generally accepted that a significant portion of the cellular creatine kinase (CK,[1] EC 2.7.3.2) in the cardiac muscles of a variety of species of animals is in the mitochrondria (Jacobs et al., 1964; Jacobus & Lehninger, 1973; Sobel et al., 1972; Farrell et al., 1972; Scholte et al., 1973; Addink et al., 1972). However, widely divergent results have been obtained with respect to the quantitative distribution as well as to the location of the particulate enzyme in the myocardium. Thus, Jacobs et al. (1964) reported that the rat heart mitochondria contain 55% of the total organ CK. Subcellular fractionation of rat heart accompanied by careful monitoring of the fractions by means of marker enzymes led Scholte (1973a) to conclude that the mitochondrial compartment accounted for 19% whereas the myofibrils contained 33% of the total CK activity. The location of this enzyme in the

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[1] Abbreviations used: CK, creatine kinase; MM, muscle-type isoenzyme; BB, brain-type isoenzyme; MB, hybrid form of isoenzyme; MT, mitochondrial isoenzyme; Cr, creatine; PCr, phosphorylcreatine; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SMP, submitochondrial particles; 2-ME, 2-mercaptoethanol; A-P_5-A, diadenosine 5′-pentaphosphate; G6PDH, glucose-6-phosphate dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; DNFB, 2,4-dinitro-1-fluorobenzene.

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