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Reactions with the Oxidized Iron Protein of *Azotobacter vinelandii* Nitrogenase: Formation of a 2Fe Center†

Gretchen L. Anderson and James Bryant Howard*

ABSTRACT: The Fe-S center of oxidized Fe protein from *Azotobacter vinelandii* nitrogenase is decomposed by α,α'-dipyridyl in a biphasic process. In the presence of MgATP, 2 Fe are immediately removed by chelation while the additional irons are removed only after several hours. A slower biphasic Fe release also was observed in the presence of chelator alone. MgADP prevented the Fe release by chelator. An intermediate in the reaction was isolated containing 2 Fe. The visible spectrum of the intermediate was similar to that of 2Fe-2S ferredoxins (ϵ_{max} at 325, 416, and 460 nm of 16.1, 11.3, and 9.0 mM^{-1} cm^{-1}). The 2Fe form was electron paramagnetic resonance (EPR) silent until partially reduced with sodium dithionite. The EPR spectral properties were similar to 2Fe-2S ferredoxins; namely, the Fe center had resonances at $g = 2.00$, 1.94, and 1.92 which were detectable, essentially unbroadened at 70 K. The results suggest that in the oxidized (2+) state Fe protein can undergo a 4Fe to 2Fe conversion.

Dinitrogen reduction requires two enzyme components: the MoFe protein (dinitrogenase), which is the site of substrate reduction, and the Fe protein (dinitrogen reductase), which is the ATP-dependent reductant for the MoFe protein. The Fe protein is a dimer of identical subunits and generally is accepted to contain 4 Fe and 4 inorganic S that can be extruded as a single 4Fe-4S cluster (Gillum et al., 1977; Averill et al., 1978). Recently, we found cysteinyl residues 97 and 132 in reduced Av2[1] subunits to be the four terminal Fe ligands (Hausinger & Howard, 1983). This is consistent with a symmetrical arrangement of the Fe cluster between subunits. In contrast, Braaksma et al. (1983) report 8 Fe and 8 S for the fully active Av2 dimer. At present, it is difficult to reconcile their values with the activity, Fe quantitation, and proposed ligands given by others [see Burgess (1984) for a comparison of the literature].

The Fe protein, upon binding MgATP, appears to undergo a conformational change as measured by shifts in the electron paramagnetic resonance (EPR) spectra, magnetic circular dichroic spectra, and redox potential (Zumft et al., 1973, 1974; Stevens et al., 1979). The most striking effect of nucleotide binding is the altered Fe center reactivity with chelators (Walker & Mortenson, 1974; Ljones & Burris, 1978; Hausinger & Howard, 1983). For example, α,α'-dipyridyl with MgATP removed 4 mol of Fe/mol of reduced Av2 ($t_{1/2} \sim 3$ min), whereas α,α'-dipyridyl with MgADP or alone complexed less than 0.2 Fe/mol after 5 h (Hausinger & Howard, 1983). For the reversibly or irreversibly oxidized protein, Walker & Mortenson (1974) and Ljones & Burris (1978) found the Fe center was chelated independent of MgATP. As part of our studies attempting to identify the specific thiol ligands in different Av2 oxidation states, we repeated these experiments. In this paper, we report both qualitative and quantitative differences with the previous work on oxidized Fe protein. Our results indicate a selective abstraction of half the Fe from reversibly oxidized Av2. The chelation is prevented by MgADP and is enhanced by MgATP. The modified protein containing half the original Fe has visible and EPR spectra characteristic of a 2Fe-2S ferredoxin. Our results suggest the 4Fe-Av2 Fe-S center has different properties from those of the 4Fe-4S centers of bacterial ferredoxins or aconitase, which can undergo 4Fe → 3Fe interconversions (Kent et al., 1982; Moura et al., 1982). Furthermore, the Av2 protein structure probably has different constraints on the Fe-S center in the reversibly oxidized and reduced states.

Materials and Methods

Nitrogenase enzymes were isolated from *Azobacter vinelandii* (ATCC 13705) by a modification of a procedure described by Burgess et al. (1980). Av2, assayed at 30 °C and saturating MoFe protein, typically had a specific activity of ~2200 $nmol \cdot min^{-1} \cdot mg^{-1}$ for acetylene reduction and ~3200 $nmol \cdot min^{-1} \cdot mg^{-1}$ for hydrogenase activity (Shah et al., 1972). Protein concentrations for all experiments were determined by amino acid analysis using the known composition for Av2 (Hausinger & Howard, 1982). [Protein determination by the Hartree method gave variable results including ~60% overestimation of protein in some cases (Hartree, 1972).] All procedures were performed under a purified Ar atmosphere with a Schlenk gas manifold for transfer of solutions and

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[1] Abbreviations: Av2, Fe protein from *Azotobacter vinelandii* nitrogenase; 4Fe-Av2 and 2Fe-Av2, 4Fe and 2Fe forms of Av2; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance.

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gas-tight syringes for small volumes.

Ultraviolet and visible spectra were recorded on a Beckman DU8 spectrophotometer using double-septum, anaerobic cuvettes. (Reduced Av2 was >90% active after 5 h in the spectrophotometer.) EPR spectra were recorded in the laboratory of Dr. J. Lipscomb on a Varian E-109 spectrometer equipped with a 30-dB micropower attenuator, an Oxford Instruments ESR-10 liquid helium cryostat for temperature regulation, and digital data storage (Lipscomb & Salo, 1983). Samples, in matched tubes, were frozen and stored in liquid N_2. For spin quantitation, the area of a double integrated spectrum was compared to that of reduced spinach ferredoxin with no corrections of g-value anisotropy. Results were calculated for spectra recorded at nonsaturating microwave power and under similar conditions.

The Fe content of Av2 was determined by three different methods of release: (a) Fe released by acid hydrolysis (6 N HCl, 110 °C, 24 h under vacuum), (b) Fe released by acid denaturation (pH 2.0, 30 min), and (c) Fe released by MgATP-dependent chelation (Walker & Mortenson, 1974; Ljones & Burris, 1978; Hausinger & Howard, 1983). The Fe was quantified by the color reaction with α,α'-dipyridyl (ϵ = 8.4 cm^{-1} mM^{-1} at 520 nm; Walker & Mortenson, 1974) or o-bathophenanthrolinedisulfonate (ϵ = 22.1 cm^{-1} mM^{-1} at 535 nm; Ljones & Burris, 1978). Buffer from the final Av2 purification step was treated in the same way and used as a reagent blank. The correction was ~15% of the total Fe. For the material used in this work, 3.8–4.8 mol of Fe/mol of Av2 was found by methods a and b while a ratio of 3.8–4.3 was found for method c.

Oxidized Av2 was prepared with extraordinary precautions to exclude O_2. All manipulations were performed on a Schlenk manifold inside of a Coy anaerobic tent (90% Ar/10% H_2 atmosphere over Pd catalyst). Highly purified Ar was further deoxygenated by passing the gas through a sodium dithionite solution and buffer trap. Sodium dithionite was removed from reduced Av2 by anaerobic chromatography on DEAE-Sepharose (pH 8.0, 50 mM Tris-HCl buffer; sodium dithionite elutes at ~0.25 M NaCl while Av2 elutes at ~0.5 M NaCl). The pooled protein was incubated with oxidized indigo carmine (0.25 mM final concentration, ~5-fold excess over protein) for 30 min at 20 °C. Oxidation of the protein could be monitored by the absorbance loss of the dye at 608 nm. The protein was separated from the dye by chromatography on DEAE-Sepharose. Repeated cycles of oxidation and reduction resulted in ~5% loss of activity per cycle.

Results and Discussion

The Fe-S center of reduced Fe protein is susceptible to chelation only in the presence of MgATP (Walker & Mortenson, 1974; Ljones & Burris, 1978; Hausinger & Howard, 1983). This has been cited as evidence for a nucleotide-dependent conformational change. In addition, Walker & Mortenson (1974) and Ljones & Burris (1978) reported a rapid nucleotide-independent release of Fe from oxidized Fe protein. We have repeated the α,α'-dipyridyl Fe release experiments, and the results for both oxidized and reduced Av2 are shown in Figure 1. Although we confirm some features of the earlier studies, several differences were noted. First, Fe release from oxidized Av2 with α,α'-dipyridyl (±MgATP) was biphasic. In the presence of MgATP, the rate of the rapid phase for oxidized Av2 exceeded that for reduced Av2; however, only ~67% of the potential absorbance change occurred in the initial burst (see Figure 1). The reaction without MgATP was similar to that with MgATP, albeit slower. Second, as for reduced Av2, MgADP prevented Fe chelation.

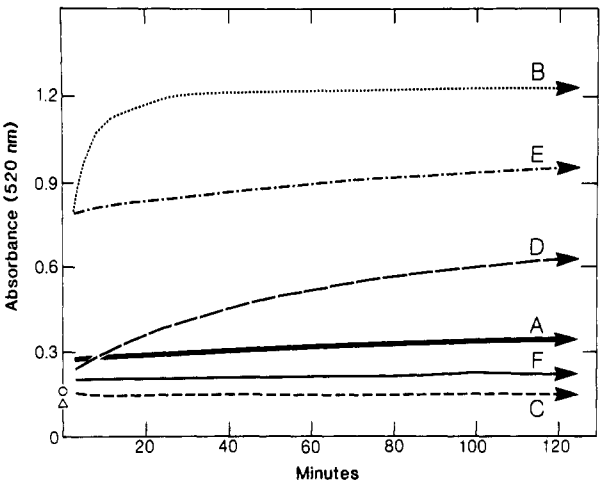

FIGURE 1: Absorbance at 520 nm for Av2 and 4 mM α,α'-dipyridyl in pH 8.0, 50 mM Tris-HCl buffer containing 0.1 M NaCl at 25 °C. The protein concentrations were 28–38 μM but are normalized to 30.0 μM for graphic representation. (A) Reduced Av2; (B) reduced Av2 + MgATP; (C) reduced Av2 + MgADP; (D) oxidized Av2; (E) oxidized Av2 + MgATP; (F) oxidized Av2 + MgADP. Nucleotide concentrations were 5 mM.[4] (Δ) is the initial absorbance for reduced Av2 and (O) is that of oxidized Av2 (both are corrected for reagent dilution).

In addition to the possibility that the Fe was selectively removed, two alternate explanations for the apparent biphasic Fe release were considered.

(a) The protein might be in a mixture of oxidation states, some of which were susceptible to chelation and others not. We believe this is not the case and only reversibly oxidized Av2 was being studied. The protein had 90–100% of the original activity after oxidation, the oxidation–reduction cycle could be repeated several times with minimal activity loss, and the protein had the appropriate EPR and visible spectral properties for reversibly oxidized Av2 (see below). Furthermore, we have found that irreversibly oxidized Av2 has different properties than those reported here.[2]

(b) The 4Fe-4S center of oxidized Av2 presumably is in the 2+ state, which is composed of 2 Fe^{2+} and 2 Fe^{3+}. Although both Fe^{2+} and Fe^{3+} are chelated by α,α'-dipyridyl, the colorimetric reaction is only for the ferrous state; thus, only half of the Fe might be observed in the absence of reductant.[3] To confirm that the measured absorbance change reflected total Fe released, free Fe and protein-bound Fe were estimated after separation by chromatography on Sephadex G-25. In Figure 2 are shown the results for two reaction conditions. As predicted from the absorbance change (Figure 1, A), little Fe was released by α,α'-dipyridyl from reduced Av2. Likewise, as predicted from Figure 1, E, 50% of the Fe was released in 10 min from oxidized Av2 with MgATP and α,α'-dipyridyl. For the slower reaction without MgATP, several samples at various times were analyzed by chromatography, and the results are shown in Figure 3. The release of Fe as analyzed by chromatography appears to follow a similar time course as that observed directly by absorbance change.

The results presented above are consistent with a biphasic release of Fe with an intermediate form of Av2 having 2 Fe. To study the properties of putative 2Fe-Av2, oxidized Av2 was treated with 4 mM α,α'-dipyridyl and 5 mM MgATP[4] for 10

[2] G. Anderson and J. B. Howard, unpublished results.

[3] Because 4 Fe^{2+} ultimately were detected by α,α'-dipyridyl, there must be a reductant in the reaction. Inorganic sulfide released from the Fe-S center is the likely source. In a separate experiment, we have shown that 2 equiv of S^{2-} will reduce 1 equiv of Fe^{3+}-α,α'-dipyridyl.

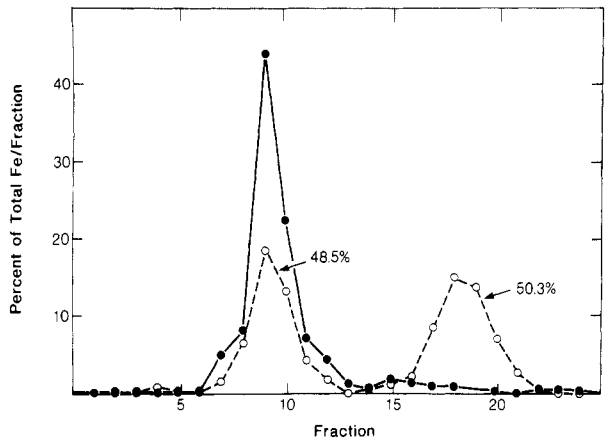

FIGURE 2: Anaerobic chromatography of Av2 on Sephadex G-25 (1 × 22 cm) in pH 8.0, 50 mM Tris-HCl buffer containing 0.1 M NaCl. (●) Reduced Av2 with α,α'-dipyridyl; (○) oxidized Av2 incubated with α,α'-dipyridyl and 5 mM ATP[2] for 10 min. To a 500-μL portion of each fraction (1.0 mL) was added 30 μL of 1 M HCl to release protein-bound Fe. After 30 min, the sample pH was adjusted to 8.0 with 60 μL of 1 M Tris base. To measure the Fe, 100 μL of 12 mM α,α'-dipyridyl and 10 μL of 1 M sodium dithionite were added, and the absorbance at 520 nm was read.

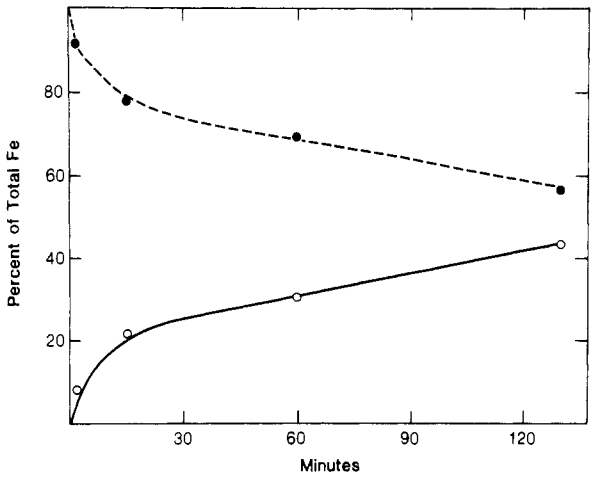

FIGURE 3: Release of Fe from oxidized Av2 by α,α'-dipyridyl. Reaction conditions as in Figure 1. At various times, samples were removed and the free Fe (○) was separated from the protein-bound Fe (●) as in Figure 2.

min and excess reagents were removed by gel filtration. The protein was devoid of activity in the acetylene reduction assay. The visible spectrum for 2Fe-Av2 (as isolated) is shown in Figure 4. The spectrum is distinctly different from that of reduced or oxidized 4Fe-Av2[5] but resembles that of oxidized 2Fe-2S ferredoxins [see Orme-Johnson (1973)]. For example, 2Fe-Av2 has ϵ_{max} at 325, 416, and 460 nm of 16.1, 11.3, and 9.0 mM^{-1} cm^{-1}, respectively; putidaredoxin has ϵ_{max} at 325, 415, and 455 nm of 15.0, 10.0, and 9.6 mM^{-1} cm^{-1}, respectively (Tsibris & Woody, 1970). The spectrum observed for 2Fe-Av2 is consistent with the removal of 2 Fe^{2+} from the 2+ state of a 4Fe-4S center in oxidized Av2. Upon reduction with excess sodium dithionite, the spectrum became a broad, featureless shoulder typical of reduced Fe-S proteins [ϵ(420 nm) = 5.1 mM^{-1} cm^{-1}]. If 2Fe-Av2 was cycled between reduced and oxidized states, the oxidized spectrum was shifted to the red 5–7 nm but had the same extinction coefficients as the

[4] The concentration of reagents was 6 mM $MgCl_2$ and 6 mM ATP, which calculates to be 5 mM MgATP (Frey et al., 1972).

[5] $\Delta\epsilon$(ox–red, 430 nm) = 7.5 mM^{-1} cm^{-1} for 4Fe-Av2, which compares favorably with the value 6.6 mM^{-1} cm^{-1} predicted by Ljones & Burris (1978) from partially active material.

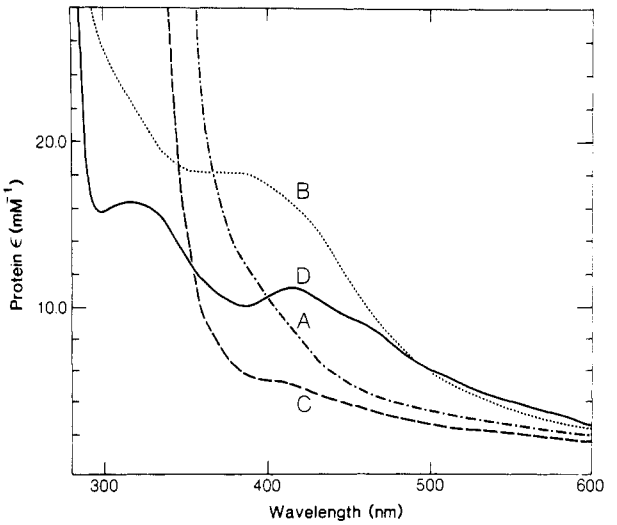

FIGURE 4: Ultraviolet–visible spectra for Av2 in pH 8.0, 50 mM Tris-HCl buffer containing 0.1 M NaCl at 25 °C. (A) Reduced 4Fe-Av2; (B) oxidized 4Fe-Av2; (C) reduced 2Fe-Av2; (D) oxidized 2Fe-Av2.

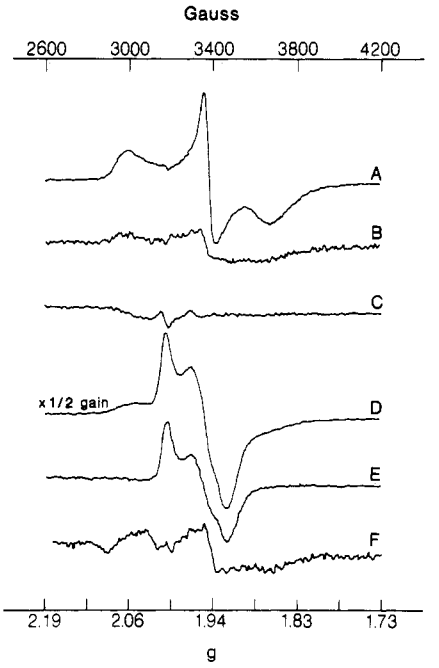

FIGURE 5: EPR spectra of Av2. Spectra are normalized to 100 μM protein (actual values 85–125 μM) and 2 × 10^4 gain. All spectra were recorded with the same instrument conditions: scan time, 8 min; time constant, 0.25 s; modulation amplitude, 10 G; modulation frequency, 100 Hz. (A) Reduced 4Fe-Av2, 15 K, 40 μW; (B) same as (A) at 35 K; (C) oxidized 2Fe-Av2, 15 K, 200 μW; (D) partially reduced 2Fe-Av2 (0.5 equiv of sodium dithionite), 20 K, 200 μW; (E) same as (D) at 68 K; (F) 2Fe-Av2 with excess sodium dithionite, 30 K, 200 μW.

2Fe-Av2 originally isolated. Although no Fe was lost with redox cycling, the spectral shift indicates a ligand rearrangement or formation of a new center.

The different states of Av2 were characterized by their EPR spectra as shown in Figure 5. Reduced 4Fe-Av2 had a rhombic spectra with g (av) <2. The signal intensity readily saturated below 12 K and disappeared due to relaxation effects above 35 K (Figure 5). Oxidized 4Fe-Av2 was EPR silent from $g \cong 9$ to 1.7 (figure not shown). These properties are consistent with the proposed Av2 4Fe-4S center cycling between 2+ and 1+ states. The reduced 4Fe-Av2 spectrum was double integrated and quantified by comparison to the spinach ferredoxin spectrum; the 4Fe-Av2 spectrum corresponded to

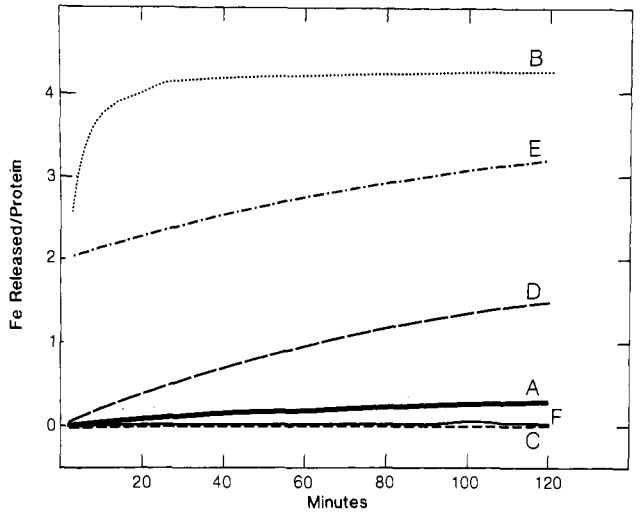

FIGURE 6: Fe release from oxidized and reduced Av2 estimated from absorbance in Figure 1. (A) Reduced Av2; (B) reduced Av2 + MgATP; (C) reduced Av2 + MgADP; (D) oxidized Av2; (E) oxidized Av2 + MgATP; (F) oxidized Av2 + MgADP. All values have been corrected for extraneous Fe (0.2–0.3 Fe/protein), which was estimated from the difference between the measured absorbance and the expected absorbance (assuming no Fe release) at 2.5 min for either oxidized or reduced Av2 + MgADP. For (A), (C), and (F) the Fe was calculated by assuming no change in protein absorbance. For (B) Fe was calculated by assuming no contribution from protein absorbance. For (D) and (E) the Fe was calculated by assuming the two-step model (see text). The rate constants were calculated by curve fitting the data in Figure 1: for (D), $k_1 = 1.1 \times 10^{-2}$ min^{-1}, $k_2 = 1.2 \times 10^{-3}$ min^{-1}; for (E), $k_1 > 10^{-1}$ min^{-1}, $k_2 = 7.9 \times 10^{-3}$ min^{-1}.

0.28–0.38 spins. The spectral shape, temperature dependence, and spin quantitation are similar to those reported by others (Orme-Johnson et al., 1972; Zumft et al., 1973; Smith et al., 1973).

2Fe-Av2, as initially isolated, was EPR silent from $g \cong 9$ to 1.7 ($g = 2$ region is shown in Figure 5). This was anticipated for a 2Fe-2S center in the 2+ state, which was indicated by the visible spectrum of 2Fe-Av2. Upon partial reduction (~0.7 equiv), a new EPR signal was observed that was more axial ($g = 2.00$, 1.94, and 1.92) and more intense than the reduced 4Fe-Av2 spectrum. The spectral shape and percent rhombicity (~40%) were characteristic of other binuclear Fe-S proteins (Blumberg & Peisach, 1974). A more diagnostic indication of a 2Fe center is the temperature dependence of the signal intensity; viz., a 2Fe EPR spectrum remains sharp at 70 K whereas a 4Fe EPR spectrum broadens and disappears above 30 K (Orme-Johnson & Orme-Johnson, 1982). As shown in Figure 5, the 2Fe-Av2 EPR spectrum had ~30% the intensity at 70 K as at 20 K and could be readily observed up to 100 K (spectrum not shown). Spectra of some samples had a small positive signal at $g = 2.06$ and a negative signal at $g = 1.86$ as might be expected for contaminating 4Fe-Av2. The latter signals had the same temperature dependence as 4Fe-Av2 and disappeared above 30 K.

Further reduction of the protein with excess sodium dithionite produced an unexpected result. Unlike other 2Fe proteins, the EPR signal disappeared. For 2Fe-2S centers, three oxidation states could be considered: 2+ (2 Fe^{3+} and EPR silent), 1+ (Fe^{2+}:Fe^{3+}, EPR active), and 0 (2 Fe^{2+}, EPR silent). Only the 2+ and 1+ states have been observed in proteins or model compounds; the 0 state is unstable and probably has a redox potential below that of sodium dithionite. However, the properties of the 2Fe-Av2 EPR spectra are consistent with the three-state hypothesis. On the other hand, the visible spectrum for reoxidized 2Fe-Av2 indicated a structural change had occurred on reduction (see above), which may be responsible for the unusual EPR behavior in excess reductant. Further spectral studies will be needed to determine the origin of this anomaly. Because the EPR signal disappeared in excess reductant, the maximum signal was obtained at only ~0.5 equiv of sodium dithionite and integrated to 0.36–0.49 spins (~1.3 × reduced 4Fe-Av2). Any small contribution to the spin quantitation by 4Fe-Av2 was avoided by integrating at 35 K. By inspection of the relative proportion of $g = 2.06$ (4Fe-Av2) and $g = 2.00$ (2Fe-Av2) in a mixed sample at various stages of reduction, it appears that 2Fe-Av2 has a more positive E_0' than 4Fe-Av2. A more detailed titration has been hampered by the unusual EPR behavior.

The data presented above suggest the Fe can be released in a two-step process (Scheme I). The earlier studies on Fe release from oxidized Fe protein apparently missed the biphasic pattern, in part, due to the absorbance contribution from 2Fe-Av2. To correct for the protein absorbance, the data (Figure 1) were recalculated by assuming two pseudo-first-order steps (Scheme I). The fast and slow rate constants were determined by curve fitting. The fraction of each component was computed by numerical integration of the rate expressions using the extinction coefficients for 2Fe-Av2, 4Fe-Av2, and Fe-α,α'-dipyridyl. The results are shown in Figure 6. As replotted, the systematic release of 2 Fe from oxidized Av2 with chelator is evident.

Scheme I

$$\text{4Fe-Av2} \xrightarrow[k_1]{\text{fast}} \text{2Fe-Av2} + 2(\text{Fe}^{2+}\text{-}\alpha,\alpha'\text{-dipyridyl})$$

$$\text{2Fe-Av2} \xrightarrow[k_2]{\text{slow}} 2(\text{Fe}^{2+}\text{-}\alpha,\alpha'\text{-dipyridyl})$$

This is the first example of a 4Fe → 2Fe conversion in a protein. Although 2Fe-Av2 has the spectral properties of a binuclear Fe-S center, we have not yet determined the inorganic S content or terminal ligands of the cluster. Because the apparent molecular weight of 2Fe-Av2 by gel chromatography is the same as for 4Fe-Av2, the new Fe center also might bridge the two subunits (chromatogram not shown). The protein must provide specific constraints on the center which allows only 2 Fe (one face of the cubane 4Fe-4S center) to be exposed to chelators. This suggests the two halves of the center may behave independently of each other under some circumstances, e.g., during electron transfer. The unusual properties of the center(s) may be related to the low-spin quantitation observed with the Fe protein and may provide a mechanism for two-electron redox of the protein observed in some experiments (Thorneley et al., 1976; Braaksma et al., 1982).

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Articles

Effect of Serum Albumin on Siderophore-Mediated Utilization of Transferrin Iron†

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ABSTRACT: The effect of serum and serum proteins on enterobactin- and aerobactin-mediated utilization of transferrin iron has been investigated. Serum was found to impede transfer of iron from iron transferrin to enterobactin and from [^{55}Fe]ferric enterobactin to cells of *Escherichia coli* BN3040 Nal^{R} *iuc*. In contrast, serum had essentially no effect on the rate of these reactions mediated by aerobactin. Three purified serum proteins, human serum albumin, bovine serum albumin, and human immunoglobulin, were comparable to human serum in their selective ability to interfere with the transfer of ^{55}Fe from [^{55}Fe]ferric enterobactin to *E. coli* BN3040 Nal^{R} *iuc*. The inhibitory effect of human serum albumin on the enterobactin-mediated transfer of iron from [^{55}Fe]transferrin was enhanced by preincubation of the protein with the siderophore. Pretreatment of the bacterial cells with human serum albumin did not affect the rate of utilization of siderophore iron. A linear, reciprocal relationship was found to hold for human albumin concentration vs. the first-order rate constant (k_{obsd}) for the velocity of iron transfer from iron transferrin to enterobactin. Binding of serum albumin to enterobactin increased the intensity of the near-ultraviolet absorption band of the siderophore and shifted it to longer wavelengths. The stoichiometry of binding to human and bovine serum albumins was established as 1:1, and the binding constant for both enterobactin and ferric enterobactin was estimated to be in the range 1×10^4–1.2×10^5 M^{-1}. These results indicate that serum albumin may act synergistically with other factors in the serum, such as transferrin, to limit iron supply and in this way restrict the growth of invading microorganisms.

Iron is probably essential for the growth of all pathogenic bacteria. Invading organisms which reach either mucosal surfaces or the circulating plasma become exposed to the iron-binding proteins lactoferrin and transferrin. These proteins, in combination with antibodies, often have powerful bacteriostatic effects in vitro and are essential for protection against many infections (Rogers et al., 1980; Bullen, 1981). Transferrin and lactotransferrin restrict the amount of ionic iron available in body fluids to 10^{-12} μM or less. This amount is insufficient for normal bacterial growth, and the ability to sequester iron, which either is insoluble or is bound to specific proteins, has been suggested as one of the factors determining the virulence of a microorganism (Weinberg, 1978).

Enteric bacteria secrete the catechol-type siderophore enterobactin, and the ferric enterobactin complex formed in the external medium is actively transported across cell membranes

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