First Report of a 2-equiv Reduction of [2Fe−2S] Ferredoxins

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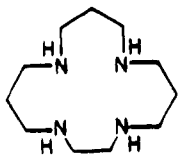
The [2Fe−2S] ferredoxins ($M_r \sim 10\,500$; 93−99 amino acids) obtained from the leaves of higher plants and from algae are an important class of electron-transfer protein involved in photosynthetic electron transport.[1−3] Reduction of the $Fe^{III}Fe^{III}$ protein by 1 equiv of a Cr^{II}−macrocycle complex, hereafter referred to as $Cr^{II}L$, generates $Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ with $Cr^{III}L$ covalently attached to the protein at a point close to the Tyr-82 residue (sometimes referred to as Tyr-83). On addition of excess $Cr^{II}L$ further reduction without attachment occurs to give Fe^{II}-$Fe^{II}\cdot\cdot\cdot Cr^{III}L$ in a redox step not previously observed and, therefore, of considerable interest.

The ferredoxins are very acidic proteins (pI 3−4),[1] with charge balance ~ -18 (± 2) at pH $\sim$ 7.5 from their amino acid compositions. Five X-ray crystal structures have been reported for the $Fe^{III}Fe^{III}$ state proteins from the blue-green algae *Spirulina platensis*,[4] *Aphanothece sacrum*,[5] *Anabaena* 7120,[6] *Halobacterium* of the Dead Sea,[7] and *Equisetum arvense*,[8] (resolutions 1.7−2.5 Å). The active site consists of two di-μ-sulfido bridged high-spin tetrahedral Fe^{III}s ($S = {}^5/_2$), which are antiferromagnetically coupled and therefore EPR silent.[9] The core is coordinated to four cysteines (RS^-) at residues 41, 46, 49, and 79 to give $[Fe_2S_2(SR)_4]^{2-}$. One of the metal atoms, Fe_A, is close to the surface (~5 Å), and the Cys-41 and Cys-46 residues bonded to it are partially exposed to solvent water. Reduction potentials for the one-electron redox process, eq 1, are -430 ± 20 mV.[10] So far no evidence for further reduction

$$Fe_2S_2^{2+} + e^- \rightleftarrows Fe_2S_2^{+} \qquad (1)$$

to the $Fe^{II}Fe^{II}$ state has been obtained even under strongly reducing electrochemical conditions. Although there is no crystal structure of the $Fe^{II}Fe^{III}$ protein, it has been demonstrated by NMR that the extra electron is localized on Fe_A.[11]

In the present studies parsley, spinach, and *A. variabilis* ferredoxins were isolated by procedures already described.[12] Air-free conditions are required for the $Fe^{II}Fe^{III}$ state and also for storage of the $Fe^{III}Fe^{III}$ protein. The latter has a characteristic UV−vis absorbance peak at 422 nm ($\epsilon = 9200\ M^{-1}\ cm^{-1}$). In each case two isoferredoxins (FdI and FdII in order of elution) are separated by FPLC using a Phenyl-Superose HR5/5 hydrophobic interaction column, and have a substantial number of amino acids different (~25).[13] The two forms exhibit similar reactivities.[14] The Cr^{II}−macrocycle complex $[Cr(15\text{-ane}N_4)(H_2O)_2]^{2+}$ was prepared by addition of 1:1 equivalents of 1,4,8,-12-tetraazacyclopentadecane (Strem Chemicals) (15-aneN_4) to an air-free solution of $CrCl_2{\cdot}4H_2O$.[15] Concentrations of the



15-aneN_4

complex were determined at the 540 nm peak ($\epsilon = 36.5\ M^{-1}\ cm^{-1}$). At pH < 1 the Cr^{III} analogue $[Cr(15\text{-ane}N_4)(H_2O)_2]^{3+}$ has peaks at 377 (88) and 454 (87), and slightly less intense peaks for the mono and bis conjugate-base forms ($H_2O \rightarrow OH^-$), acid dissociation constants $pK_a = 2.9$ and 7.8.[16] The $Cr^{III}L$/$Cr^{II}L$ couple has a reduction potential of −580 mV.[17] When excess $Cr^{II}L$ is used as reductant for the FdI protein, the product isolated by DE52 ion-exchange chromatography has only one $Cr^{III}L$ attached, in keeping with earlier work.[18] Thus the products give Cr:Fe ratios of 1:2.2 (parsley) and 1:2.0 (spinach) by ICP atomic emission spectroscopy. The Cr was also determined by a diphenylcarbazide method.[19]

In stopped-flow studies at 25 °C with $Cr^{II}L$ in large, >50-fold, excess, $I = 0.100$ M (NaCl), biphasic kinetics are observed (software from OLIS, Bogart, GA). Absorbance changes are consistent with a reaction proceeding farther than the $Fe^{II}Fe^{III}$ stage, which is the product observed on reaction with, e.g., dithionite.[20] The final spectrum obtained on consumption of 2 equiv of $Cr^{II}L$ is shown in Figure 1. No contribution of $Cr^{II}L$ or $Cr^{III}L$ to these spectra was detectable. Absorbance changes at selected wavelengths (generally 422 nm) were in accordance with Figure 1, and from a standard consecutive reaction treatment first-order rate constants k_{1obs} and k_{2obs} were obtained. Both constants give a first-order dependence on [$Cr^{II}L$], Figure 2. Hence, from the slopes, second-order rate constants for parsley FdI, $k_1 = 1510\ M^{-1}\ s^{-1}$, and $k_2 = 210\ M^{-1}\ s^{-1}$, were determined at pH 7.5 (20 mM Tris/HCl). Similar behavior was observed for spinach FdI, but *A. variabilis* FdI gave only the first stage of reaction, and no $Cr^{III}L$ was attached to the product. On decreasing the pH in the range 8.5−5.0 both k_1 and k_2 give large, >10-fold, increases, but with no leveling out, consistent with a single effective protonation step. The reaction sequence can be expressed as in eq 2.

$$Fe^{III}Fe^{III} \xrightarrow[k_1]{+Cr^{II}L} Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L \xrightarrow[k_2]{+Cr^{II}L} Fe^{II}Fe^{II}\cdot\cdot\cdot Cr^{III}L + Cr^{III}L \qquad (2)$$

On rapid reoxidation of $Fe^{II}Fe^{II}\cdot\cdot\cdot Cr^{III}L$ with $[Co(NH_3)_6]^{3+}$, it was demonstrated that after ~40 min some 80% of the original $Fe^{III}Fe^{III}$ absorbance was restored, Figure 1. There is, however,

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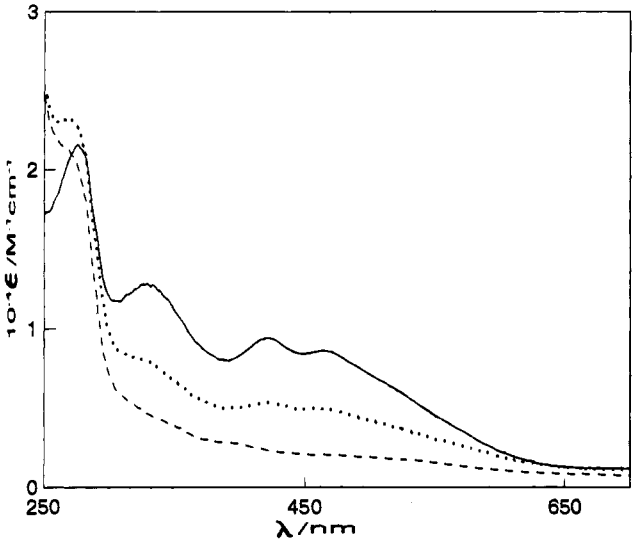

Figure 1. UV−vis spectra of the $Fe^{III}Fe^{III}$ (—), $Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ (•••), and $Fe^{II}Fe^{II}\cdot\cdot\cdot Cr^{III}L$ (- - -) forms of parsley and spinach [2Fe−2S] ferredoxins generated by reduction with $[Cr(15\text{-aneN}_4)(H_2O)_2]^{2+}$ at pH 7.5 (20 mM Tris/HCl), I = 0.100 M (NaCl). The spectrum of $Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ is similar to that of the $Fe^{II}Fe^{III}$ protein, with $Cr^{III}L$ making a negligible contribution.

a slow denaturation of the $Fe^{II}Fe^{II}$ state which in addition is very air sensitive. From cyclic voltammetry and square wave voltammetry on purified $Fe^{III}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ were obtained potentials of −409 and −277 mV (vs NHE).[21] Two one-electron steps are therefore defined as in eq 3.

$$Fe^{III}Fe^{III}\cdot\cdot\cdot Cr^{III}L \rightleftharpoons Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L \rightleftharpoons Fe^{II}Fe^{II}\cdot\cdot\cdot Cr^{III}L \quad (3)$$

In other experiments it was demonstrated that $Fe^{II}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ is reduced to $Fe^{II}Fe^{II}\cdot\cdot\cdot Cr^{III}L$ by dithionite ($Na_2S_2O_4$ from Merck). The $Fe^{II}Fe^{III}$ protein is not, however, reduced by $Cr^{II}L$. The presence of attached $Cr^{III}L$ is therefore critical for the second stage of reduction as in eq 2. It was also shown that $Fe^{III}Fe^{III}\cdot\cdot\cdot Cr^{III}L$ is rereduced by 2 equiv of $Cr^{II}L$, but the rate constants are smaller. Indeed the rate constant observed for the first stage now corresponds closely with that previously observed for k_2. Furthermore, in the reaction of the $Fe^{III}Fe^{III}$ protein with $Cr^{II}L$, redox inactive $[Cr(en)_3]^{3+}$ gives competitive inhibition for k_1 but not k_2. At levels of $[Cr(en)_3]^{3+}$ giving maximum inhibition, rate constants for the first stage converge on k_2. One possible interpretation is that there are two reaction sites, one of which is blocked by the attached $Cr^{III}L$ or by $[Cr(en)_3]^{3+}$. For physiological reactions involving redox interconversion of $Fe^{III}Fe^{III}$ and $Fe^{II}Fe^{III}$, two such sites may be involved as has been proposed for plastocyanin in its reactions with cytochrome f and $P700^+$.[22]

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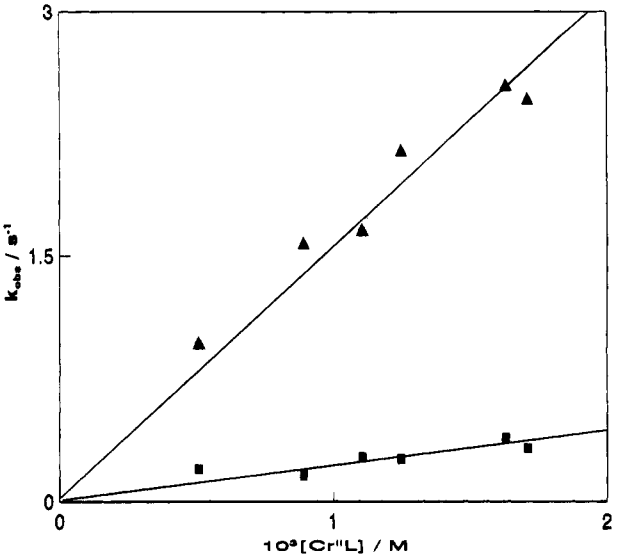

Figure 2. Thd dependence of first-order rate constants k_{obs} (25 °C) for the first (▲) and second (■) stages of the reduction of parsley [2Fe−2S] FdI by $[Cr(15\text{-aneN}_4)(H_2O)_2]^{2+}$ at pH 7.5 (20 mM Tris/HCl), I = 0.100 M (NaCl).

From NMR studies on $Fe^{III}Fe^{III}\cdot\cdot\cdot Cr^{III}L$, ^1H line broadening of Tyr-82 by the paramagnetic $Cr^{III}L$ is observed. The Tyr-25 is too close to the $Fe^{III}Fe^{III}$ site for similar observations to be made. Relevant 2D studies are in progress. Evidence has been obtained previously for a reaction site close to the surface exposed/stacked phenolic acid rings of Tyr-25 and Tyr-82 (4.2 Å separation).[23] The aromatic ring of Tyr-25 is 3.35 Å from S(Cys-79), which is the nearest point of the active site. The corresponding "through-bond" distance using the pathways program[24] is 9.4 Å. EPR spectra show a number of interesting features, which are being further explored.

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