Mechanism of Formation, Spectrum and Reactivity of Half-Reduced Eight-Iron *Clostridium pasteurianum* Ferredoxin in Pulse-Radiolysis Studies and the Non-Co-operativity of the Four-Iron Clusters

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Reduction of fully oxidized *Clostridium pasteurianum* 8-Fe_{ox.,ox.} ferredoxin by using pulse-radiolysis techniques yields the half-reduced species 8-Fe_{ox.,red.} ferredoxin. The subsequent oxidation of 8-Fe_{ox.,red.} ferredoxin with Co(NH₃)₅Cl²⁺ was studied. From a comparison with stopped-flow studies on the 2:1 Co(NH₃)₅Cl²⁺ oxidation of 8-Fe_{red.,red.} ferredoxin to the 8-Fe_{ox.,ox.} form it is concluded that there is no redox co-operativity between the two 4-Fe centres in these reactions.

Structural features (Adman *et al.*, 1973; Carter, 1976) as well as amino acid sequences (Yasunobu & Tanaka, 1973) of 8-Fe ferredoxins (mol.wt. approx. 6000) are now fairly well established. Two Fe₄S₄^{*} cubane-like clusters (where S^{*} denotes sulphide) are bound to a single peptide chain by cysteine residues, and are positioned about 1.2 nm apart at either end of a cavity. Each 4-Fe cluster contains equivalent Fe atoms (average oxidation state 2.5 for the oxidized form), and is one-electron redox active (reduction potential about -0.40 V) (Stombaugh *et al.*, 1976). An approximate twofold symmetry has been observed in the crystal structure of the *Pseudomonas aerogenes* 8-Fe ferredoxin, in which each half of the polypeptide chain binds three cysteinyl sulphur atoms to one cube and another cysteinyl sulphur atom to the other. This arrangement could conceivably lend itself to redox co-operativity between the two 4-Fe cubes. We here report studies on *Clostridium pasteurianum* 8-Fe ferredoxin that relate to this question.

Methods and Materials

Pulse-radiolysis experiments were carried out with the aid of a linear accelerator facility (Keene, 1964). Aqueous thiocyanate was used for dosimetry (Adams *et al.*, 1965). For reactions of the hydrated electron e_{aq.}⁻, anaerobic conditions (Ar-saturated solutions) were made up with 1.0 mM-potassium phosphate buffer, pH 7.0, and 0.10 M-NaClO₄. 2-Methylpropan-2-ol (0.2 M) was used to scavenge OH^{*} and H^{*} radicals (eqn. 1):

The 2-methylpropan-2-ol radicals play no further part in the reaction (Farragi & Tal, 1975; De Kok *et al.*, 1977). Formate radicals were obtained by pulsing N₂O-saturated solutions of 1.0 mM-potassium phosphate buffer, pH 7.0, and 0.10 M-sodium formate. In these solutions the hydrated electrons are converted into OH^{*} radicals by reaction with N₂O (Brown *et al.*, 1965) (eqn. 2):

and OH^{*} and H^{*} radicals react with formate ions (Buxton & Sellers, 1973) (eqn. 3):

The propan-2-ol radical reaction was investigated by using N₂O-saturated solutions of 1.0 mM-phosphate buffer, pH 7.0, 0.50 M-propan-2-ol and 0.10 M-NaClO₄ (Simic *et al.*, 1969). Hydrated electrons react as in eqn. (2), whereas OH^{*} and H^{*} radicals react as in eqn. (4):

Reactions (1)–(4) are all rapid, and for the conditions specified effectively scavenge the primary radical species. The 8-Fe_{ox.,ox.} ferredoxin from *C. pasteurianum* was isolated by the procedure described by Thompson *et al.* (1974), and was standardized spectrophotometrically at the 390 nm peak (ϵ 3.0 × 10⁴ M⁻¹ cm⁻¹) (Sobel & Lovenberg, 1966). The absorbance ratio A₃₉₀/A₂₈₅ = 0.80 was used as a criterion of purity (Stombaugh *et al.*, 1976). Concentrations of ferredoxin were in the range 6–20 μM. Radical concentrations (0.5–2.0 μM) were kept below 10% of this concentration to ensure that the 8-Fe protein

is singly reduced. First-order kinetic plots of absorbance changes $\log(A_t - A_\infty)$ against time were linear to 3–4 half-lives, and rate constants $k_{\text{obs.}}$ were obtained from slopes ($\times 2.303$).

A Durrum–Gibson stopped-flow spectrophotometer was used to study the reaction of 8-Fe_{red.,red.} ferredoxin (obtained by standard procedures with Na₂S₂O₄ as a reducing agent) with Co(NH₃)₅Cl²⁺. All experiments were at 18°C and at pH 7.0 (1.0 mM-phosphate buffer) and 1.010 (NaClO₄).

Results

The reaction of e_{aq.}⁻ with 8-Fe_{ox.,ox.} ferredoxin was monitored at the e_{aq.}⁻ peak at 720 nm (ϵ 18 500 M⁻¹·cm⁻¹) (Swallow, 1973, p. 92), when a rate constant (mean $\pm$ s.d.) of $3.4 (\pm 0.2) \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ was obtained. Only small absorbance changes were observed at wavelengths in the range 260–500 nm at which the ferredoxin absorbs. These changes are small (<15%) compared with, for example, the change in absorbance observed at 420 nm for the conversion of 8-Fe_{ox.,ox.} ferredoxin into the 8-Fe_{red.,red.} form. However, at 420 nm a slower second stage, giving a significant change in absorbance corresponding to the formation of the half-reduced species 8-Fe_{ox.,red.} ferredoxin, was observed, with a rate constant of $1.1 (\pm 0.1) \times 10^3 \text{ s}^{-1}$. The radicals CO₂^{-•} and (CH₃)₂C[•]OH were also used to generate 8-Fe_{ox.,red.} ferredoxin. Because these radicals do not have a suitable absorbance, the first stage could not be monitored. The second stage, resulting in the generation of 8-Fe_{ox.,red.} ferredoxin, was observed in

Fig. 1. Absorption differences produced on reaction of 8-Fe_{ox.,ox.} ferredoxin (15 μM) solutions with e_{aq.}⁻ (▼), CO₂^{-•} (●) and (CH₃)₂C[•]OH (■) (15 μM) in a 2.5 cm cell. The continuous line represents the difference spectrum expected assuming the spectrum of 8-Fe_{ox.,red.} ferredoxin is midway between that of the 8-Fe_{ox.,ox.} and 8-Fe_{red.,red.} species.

Fig. 2. Dependence of first-order rate constants ($k_{\text{obs.}}$) on $[\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}]$ for the $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ oxidation of 8-Fe_{ox.,red.} ferredoxin generated by reaction of e_{aq.}⁻ with 8-Fe_{ox.,ox.} ferredoxin at 18°C at pH 7.0 (10 mM-phosphate buffer), 1.010 M (NaClO₄).

each case, and the same rate constant as reported above was obtained.

Details of spectra obtained after completion of the second stage are recorded in Fig. 1. For all three reductants the half-reduced species gives the same isobestic point as 8-Fe_{ox.,ox.} ferredoxin and 8-Fe_{red.,red.} ferredoxin at 286 nm. It is also concluded from the incremental changes at each wavelength that the spectrum of 8-Fe_{ox.,red.} ferredoxin is midway between that of 8-Fe_{ox.,ox.} ferredoxin and 8-Fe_{red.,red.} ferredoxin. The efficiency of reduction is less than 100%, however. The 8-Fe_{ox.,red.} species generated is stable for at least 2 s.

The rate constant for the reaction of e_{aq.}⁻ with Co(NH₃)₅Cl²⁺ was determined under present conditions to be $2.5 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ (see also Baxendale *et al.*, 1965). When a solution containing both 8-Fe_{ox.,ox.} ferredoxin (15 μM) and Co(NH₃)₅Cl²⁺ (range 11–58 μM) was pulsed the subsequent reaction of 8-Fe_{ox.,red.} ferredoxin with excess of Co(NH₃)₅Cl²⁺ could be monitored at 420 nm. A plot of first-order rate constants ($k_{\text{obs.}}$) against $[\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}]$ (Fig. 2) gives from the slope a second-order rate constant of $5.1 (\pm 0.4) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. From stopped-flow studies on the reaction of 8-Fe_{red.,red.} ferredoxin (3.6 μM) with Co(NH₃)₅Cl²⁺ (range 51–150 μM) a single amplitude change is observed and a rate constant of $4.1 (\pm 0.1) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ obtained.

Discussion

The reduction of 8-Fe_{ox.,ox.} ferredoxin with e_{aq.}⁻ occurs in two stages, with rate constants $3.4 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.1 \times 10^3 \text{ s}^{-1}$ respectively. With reductants CO₂²⁻ and (CH₃)₂C'OH the same second stage is observed, yielding 8-Fe_{ox.,red.} ferredoxin. The second stage is therefore believed to be an intramolecular process. Low overall efficiencies of reaction of e_{aq.}⁻ and CO₂²⁻ (30–40%) and of (CH₃)₂C'OH (56%) with 8-Fe_{ox.,ox.} ferredoxin are observed (see Fig. 1), where the dependence is possibly the reverse of reduction potentials for e_{aq.}⁻ (–2.9 V) (Swallow, 1973, p. 150), CO₂²⁻ (–2.0 V) (Breitenkamp *et al.*, 1976) and (CH₃)₂C'OH (–1.8 V) (Breitenkamp *et al.*, 1976). One possible interpretation is that the first stage corresponds to uptake of an electron by one of the 4-Fe centres (small absorbance change), which then undergoes reorganization/conformational change to give the final product (major absorbance change). Alternatively, the first stage may correspond to indiscriminate reduction of amino acids in the peptide chain, where many of the electrons do not find their way to a 4-Fe centre in the second stage. The second stage gives good first-order kinetics, and there is no evidence for concurrent processes each having a different rate constant. Reactions of e_{aq.}⁻ with free amino acids at approx. pH 7.0 were studied. Rate constants for tyrosine and phenylalanine, i.e. amino acids with aromatic side chains, are greater (approx. $3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) than those for other amino acids ($< 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) (Anbar *et al.*, 1973). One possibility therefore is that for the reaction yielding 8-Fe_{ox.,red.} ferredoxin the first stage corresponds to reduction of either the tyrosine or phenylalanine in the protein (Adman *et al.*, 1973; Carter, 1976; Yasunobu & Tanaka, 1973), and that the second stage consists of electron transfer to one of the 4-Fe clusters. By analogy with the *Ps. aerogenes* structure the tyrosine and phenylalanine residues present in the *C. pasteurianum* 8-Fe protein are situated one close to each 4-Fe unit.

Pulse-radiolysis studies on the 2-Fe pea ferredoxin indicate a similar two-stage process for the reaction of the Fe_(III,III) form with e_{aq.}⁻. A rate constant of $4 \times 10^3 \text{ s}^{-1}$ has been reported for the second stage (Blyumenfeld *et al.*, 1977). Bielski (1977) in a preliminary report on the reactions of spinach 2-Fe ferredoxin reports only a first-stage rate constant, approx. $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$.

We now turn to the oxidation of 8-Fe_{red.,red.} ferredoxin by excess of Co(NH₃)₅Cl²⁺, which can be represented by two one-electron steps (eqns. 5 and 6):

For mild redox partners, such as Co(III), we have no information as to whether electron transfer is or is not a two-stage process similar to that identified above. The rate constant k_2 from pulse-radiolysis studies is $5.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. From stopped-flow studies an overall rate constant of $4.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ is obtained. For the latter study a single exponential was observed with absorbance change corresponding to the transformation of 8-Fe_{red.,red.} ferredoxin into the 8-Fe_{ox.,ox.} form. This is consistent with $k_2 \gg k_1$ or, since equal $\Delta\epsilon$ increments are observed for the two steps, $k_2 \approx k_1$. By using a consecutive reaction treatment, the overall rate constant from stopped-flow studies and k_2 from pulse-radiolysis experiments, a best fit of $k_1 = 7.7 \times 10^5 \text{ s}^{-1}$ was obtained. Clearly therefore the condition $k_2 \gg k_1$ does not apply and $k_1 \approx k_2$. Thus significantly more rapid oxidation by the second Co(NH₃)₅Cl²⁺, i.e. co-operativity, does not exist in this system, and the results obtained support the reduction potentials of 0.367 and 0.398 V reported (Eisenstein & Wang, 1969) for the two 4-Fe units of an 8-Fe protein.

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