Formation of super-reduced *Chromatium* high-potential iron–sulphur protein in aqueous solution by pulse radiolysis

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Both the oxidized and reduced forms of Hipip (high-potential iron–sulphur protein) are reduced (approx. 30% yields) by e_{aq}^- in a single-stage process, rate constants 1.7×10^{10} and $1.8 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively, at 25°C, pH 7.0 (5 mM-phosphate). Super-reduced Hipip, which is formed in the latter case, has a spectrum which closely resembles that of reduced ferredoxin, i.e. $\text{Fe}_4\text{S}_4^*(\text{SR})_4^{3-}$ clusters. The spectrum is stable over 2 s periods investigated. Super-reduced Hipip is reoxidized with O_2 , rate constant $4.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25°C.

Chromatium vinosum D Hipip has been the subject of extensive studies (Averill & Orme-Johnson, 1978). It contains a single cubane-like 4-Fe cluster, $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ (where S^* represents inorganic sulphide and SR a cysteine amino-acid residue), associated with a single polypeptide chain, and has a molecular weight of approx. 9500 (Dus *et al.*, 1973). Crystal structure determinations have been reported (Carter, 1977 and references therein.). Unlike ferredoxins containing $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ clusters, which have reduction potentials of approx. –400 mV (Stombaugh *et al.*, 1976), Hipip has a reduction potential of 350 mV (Dus *et al.*, 1967), also for a one-electron change (Mayhew *et al.*, 1969). This striking difference has been rationalized by a ‘three-state’ hypothesis (Carter, 1977), in which it has been proposed that, whereas the ferredoxins operate between charged clusters $\text{Fe}_4\text{S}_4^*(\text{SR})_4^{3-}$ (average Fe oxidation states 2.5 and 2.25, respectively), the Hipip proteins operate between $\text{Fe}_4\text{S}_4^*(\text{SR})_4^{2-}$ (average Fe oxidation states 2.75 and 2.5 respectively). Why the two classes of protein should exhibit this different redox behaviour is not clear. The number of NH–S hydrogen bonds (Adman *et al.*, 1975), and/or involvement of a conformational change are possible explanations.

Strong reducing agents such as Cr(II)–EDTA (–100 mV) appear to be without effect on reduced Hipip (Henderson & Sykes, 1980). A super-reduced

form has been obtained by dithionite reduction, but the protein has to be in an unfolded state in 80% dimethyl sulphoxide before this reaction takes place (Cammack, 1973). In the present paper the hydrated electron, e_{aq}^- (reduction potential –290 mV), generated by pulse-radiolysis of water, is used to obtain the super-reduced form. The manner of formation, spectrum, stability and reactivity are considered. Comparisons are also made with the e_{aq}^- reduction of oxidized Hipip. The different oxidation states of Hipip are denoted by Hipip_o , Hipip_r and Hipip_{sr} for oxidized, reduced and super-reduced forms respectively.

Materials and methods

Hipip was obtained in the reduced form from cells of *Chromatium vinosum* D (Microbiological Products, P.H.L.S., Porton, Wilts., U.K.) by a modification of the method of Bartsch (1971). After purification, samples gave an absorbance peak ratio $A_{283}/A_{388} = 2.52$ –2.58, in satisfactory agreement with the literature value of 2.52 (Bartsch, 1971). Solutions were stored under N_2 and standardized spectrophotometrically (ϵ $1.61 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 388 nm). The oxidized form of the protein (ϵ $2.0 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 348 nm) was prepared by reaction with $\text{Fe}(\text{CN})_6^{3-}$. Protein solutions were dialysed against the required buffer (5 mM-phosphate) at pH 7.0.

Pulse radiolysis experiments to generate hydrated electrons, e_{aq}^- , were carried out at the Cookridge Radiation Research Centre, Leeds, U.K., and the Centre for Fast Kinetics Research, University of

Abbreviation used: Hipip, high-potential iron–sulphur protein.

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Texas, Austin, TX, U.S.A. Reactions were under anaerobic conditions (Ar-saturated solutions) with 1% 2-methylpropan-2-ol to scavenge $\text{OH}^\bullet$ and $\text{H}^\bullet$ radicals (Farragi & Tal, 1975; de Kok *et al.*, 1977). By first saturating protein solutions with N_2O , the e_{aq}^- was converted into $\text{OH}^\bullet$ (Brown *et al.*, 1965), so that the only radicals present were those of 2-methylpropan-2-ol. It was demonstrated that there was no reaction of the latter with the Hipip in 2 s. Further experiments in the presence of excess O_2 ($2 \times 10^{-3} \text{ M}$), when $>99\%$ of e_{aq}^- reacted with O_2 (to give O_2^-) and 2-methylpropan-2-ol peroxo radicals were also formed (Simic *et al.*, 1969) demonstrated that neither of these products react with Hipip. To study the reaction of super-reduced Hipip with O_2 , the latter was introduced (before pulsing) by using an O_2/Ar mixing chamber. A concentration range $(3-20) \times 10^{-5} \text{ M}$ was investigated. Because the O_2 competes with Hipip_r for e_{aq}^- it was necessary to increase the dose as the O_2 was increased, thereby allowing a constant amount of e_{aq}^- to react with Hipip_r. In all experiments the concentration of Hipip ($10-40 \mu\text{M}$) was in large excess of e_{aq}^- ($1-5 \mu\text{M}$) to maintain pseudo-first-order conditions.

Results

Reaction of Hipip_o with e_{aq}^-

Rate constants were evaluated at 578 nm (decay of e_{aq}^- absorbance) and 410 nm (change of absorbance due to Hipip). Rate constants (1.7×10^{10}

$\text{M}^{-1} \cdot \text{s}^{-1}$ at 25°C) were the same at both wavelengths. The absorbance differences between the product of the reaction and the starting material, Hipip_o, were determined at a number of wavelengths. If the assumption is made that 25–30% reduction of the Hipip_o occurs for each e_{aq}^- generated, then the difference spectrum obtained is in very good agreement with that for Hipip_o and Hipip_r in Fig. 1 (Bartsch, 1971).

Reduction of Hipip_r with e_{aq}^-

Rate constants (25°C) at the same two wavelengths were again in good agreement and gave a value of $1.8 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$. The absorbance changes in the 310–580 nm range indicated that super-reduced Hipip is formed. The experimentally observed absorbance difference at 410 nm was equated to a molar absorbance coefficient difference of $9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$, which is the absorbance difference between reduced 4-Fe ferredoxin and Hipip_r at this wavelength (Dus *et al.*, 1967; Hong & Rabinowitz, 1970). This adjustment requires that there is again 25–30% reduction of the $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ cluster in the Hipip. Using this value it can be seen

Fig. 1. Difference in absorption coefficients (ϵ) for Hipip_r, less those for Hipip_o (solid line), and the difference spectrum observed in the reaction of e_{aq}^- with Hipip_o (experimental points)

The latter spectrum has been normalized to the known $\Delta\epsilon$ at 480 nm. Experimental conditions: pH 7.0 (5 mM-phosphate).

Fig. 2. Difference in absorption coefficients (ϵ) for Hipip_r, less those for reduced ferredoxin $\text{Fe}_4\text{S}_4^*(\text{SR})_4$, obtained by halving ϵ values for *Clostridium pasteurianum* 8-Fe ferredoxin (solid line)

●, The e_{aq}^- reduction of Hipip_r; ■, the absorbance differences between Hipip_{sr} and Hipip_r in 80% dimethyl sulphoxide (Cammack, 1973). The values for (●) and (■) have been normalized at 410 and 420 nm respectively. Experimental conditions: pH 7.0 (5 mM-phosphate).

Fig. 3. Spectra of Hipip_{sr} (---) and reduced ferredoxin $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ (—) obtained by halving ϵ values for *Clostridium pasteurianum* 8-Fe ferredoxin. The absorbance of Hipip_{sr} at 410nm has been normalized to that of reduced ferredoxin at the same wavelength ($\epsilon = 6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Fig. 4. Dependence of first-order rate constants (k) for the reaction of Hipip_{sr} with O_2 on $[\text{O}_2]$. Experimental conditions: pH 7.0 (5 mM-phosphate), 21°C .

that there is good general agreement of the two different spectra (Fig. 2) and also with that deduced from the dithionite reduction of Hipip_{r} in 80% dimethyl sulphoxide (Cammack, 1973). There are quantitative differences at 310–360nm, a result we have confirmed using two pulse radiolysis facilities. The spectrum of super-reduced Hipip is stable for at least 2s at 21°C . A comparison of the spectra of reduced ferredoxin and super-reduced Hipip is shown in Fig. 3. Formate radicals (Buxton & Sellers, 1973), obtained by pulsing N_2O -saturated solutions of 5.0mM-phosphate (pH 7.0) containing 0.1 M-sodium formate, did not reduce Hipip_{r} .

Reoxidation of super-reduced Hipip with O_2

The dependence of first-order rate constants,

k_{obs} , on $[\text{O}_2]$ is illustrated in Fig. 4. This gives a second-order rate constant for the reaction of Hipip_{sr} with O_2 of $(4.8 \pm 0.6) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 21°C . The absorbance at 410nm decays back to the original level.

Discussion

No reduction of Hipip_{r} by $\text{CO}_2^{\cdot -}$ (-2.0 V) is observed, yet the reaction with $\text{e}_{\text{aq}}^{\cdot -}$ (-2.9 V) is extremely rapid ($1.8 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$). The nature of the electron and its ability to tunnel through an energy barrier is a possible explanation. Both Hipip_0 and Hipip_{r} are reduced by $\text{e}_{\text{aq}}^{\cdot -}$ in a single-stage process, with no evidence for other accompanying rate-determining changes. The magnitude and similarity of rate constants (1.7×10^{10} and $1.8 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively) suggests that both processes are determined by the same controlling features, with rate constants close to the diffusion controlled limit.

The difference in spectra observed for the reduction of Hipip_0 to Hipip_{r} is as predicted from known spectra. The difference spectrum for Hipip_{r} to Hipip_{sr} is sufficiently like that for reduced ferredoxin (per 4-Fe unit) and Hipip_{r} , to leave no doubt that Hipip_{sr} is generated. Differences do occur at wavelengths less than 400nm, however (Figs. 2 and 3). In contrast with the spectra in Fig. 3, those of Hipip_{r} and oxidized ferredoxin have well-defined peaks at approx. 390nm with ϵ approx. $3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

A comparison of the present study with that of $\text{e}_{\text{aq}}^{\cdot -}$ and oxidized 8-Fe *Clostridium pasteurianum* ferredoxin (which contains two Fe_4S_4^* cubes, each one-electron active, average oxidation states 2.5 and 2.25 for oxidized and reduced cubes, respectively) is of interest. Efficiencies, as measured by the extent of reduction of the $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ clusters with $\text{e}_{\text{aq}}^{\cdot -}$ are 25–30% for Hipip (the present paper) and 30–40% for 8-Fe ferredoxin (Butler *et al.*, 1979). Loss of $\text{e}_{\text{aq}}^{\cdot -}$ is most likely due to non-discrimination in reduction of the protein, with only partial reduction of the clusters resulting. Although with Hipip rate constants obtained by monitoring the decay of $\text{e}_{\text{aq}}^{\cdot -}$ match those obtained by following the change in absorbance due to the $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ chromophore, with the 8-Fe protein the two differ and two stages are observed. It would appear that in the latter case the initial reaction of $\text{e}_{\text{aq}}^{\cdot -}$ with the protein is followed by a slower step corresponding to either (a) intramolecular electron transfer from a 'sink' (where the electron is held for a short time) to one of the $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ clusters, or (b) subsequent adjustment of the cluster with change in absorbance of the $\text{Fe}_4\text{S}_4^*(\text{SR})_4$ chromophore following initial uptake of an electron. It is concluded that in the case of both Hipip_{r} and Hipip_0 ,

intramolecular electron transfer (if it occurs) is relatively rapid, or that e_{aq}^- reacts directly with the cluster and cluster rearrangement is again relatively rapid.

The super-reduced Hipip is stable for the maximum time it was possible to observe (2 s). It is also clear from Fig. 4 that there is no observable $[O_2]$ -independent contribution, and that on the pulse radiolysis time-scale the super-reduced protein is stable. The super-reduced form is a strong-reductant and reacts with O_2 ($4.8 \times 10^6 M^{-1} \cdot s^{-1}$) presumably with the formation of O_2^- . Although Hipip_r appears to be regenerated quantitatively we have no confirmation that the reactivity remains unaffected, i.e. that it retains its high-potential properties.

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