"SUPER-REDUCTION" OF CHROMATIUM HIGH-POTENTIAL IRON-SULPHUR PROTEIN IN THE PRESENCE OF DIMETHYL SULPHOXIDE.

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SUMMARY

In solutions containing more than 70% dimethyl sulphoxide (DMSO) by volume, the normal reduced state of Chromatium HIPIP can be further reduced by dithionite to give a form with a decreased absorption around 400 nm and an EPR signal similar to those of the reduced ferredoxins. These changes are reversible and the native HIPIP is recovered on removal of DMSO. These observations favour the hypothesis that the four-iron cluster in ferredoxins and HIPIP can exist in three oxidation states.

The high-potential iron-sulphur protein (HIPIP) from Chromatium contains four atoms of iron and four atoms of sulphide in a structure which is very similar to those in the ferredoxin from Peptococcus aerogenes (1-3). However the HIPIP molecule differs from those of the ferredoxins in a number of physical properties. Its redox potential, +350 mV, is considerably higher than those of the ferredoxins, typically -400 mV. Moreover it shows an EPR signal with g_{av} around 2.06 in the oxidized form, and is non-magnetic in the reduced form, while the ferredoxins are non-magnetic in the oxidized form and show an EPR signal with g_{av} around 1.96 in the reduced form. In order to explain these differences Carter et al (3) proposed a "three-state" hypothesis: that the non-magnetic forms, reduced HIPIP and oxidized ferredoxin, represent an equivalent redox state, C, of the four-iron cluster. In HIPIP the four-iron cluster can undergo oxidation to a paramagnetic state C^{+}, while in ferredoxin a similar cluster can undergo reduction to another paramagnetic state C^{-}. One difficulty with this otherwise attractive hypothesis is that it is not clear why HIPIP cannot normally be reduced to state C^{-}, or ferredoxin oxidized to C^{+}; Carter et al, (3) suggested that subtle influences of the protein might be the cause.

MATERIALS AND METHODS

HIPIP, prepared from cells of Chromatium, strain D, by a modification of the method of Bartsch (4) was provided by Drs. M.C.W. Evans and K.K. Rao. The reduced protein was further purified by chromatography on a 60 x 2.5 cm column of Whatman DE-23 DEAE-cellulose in 60mM NaCl/20 mM Tris, pH 8.0, when it ran as a single band, all fractions of which had an absorbance ratio A_{388}: A_{282} between 0.37 and 0.39 in the reduced form. The fractions with the higher ratio were desalted on Sephadex G-25 in 20 mM Tris-Cl, pH 9.0 and used for this work. The homogeneity of the preparation was confirmed by polyacrylamide gel electrophoresis.

Dimethyl sulphoxide (spectroscopic grade) and other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Optical absorption measurements were made on a Pye Unicam SP800 spectrophotometer using an anaerobic cell of 1 cm path similar to that described by Dixon (5). The cell was maintained at 3°C and a slow stream of purified Ar was bubbled continuously through it.

EPR measurements were made on a Varian E-4 spectrometer. Samples, in tubes of 3.0 mm internal diameter, were cooled in a stream of cold He and the temperature measured by an iron-doped gold/chromel thermocouple.

Potentiometric titrations were carried out as described by Dutton (6). Mediators were benzyl viologen, methyl viologen, N,N'- dimethyl-2,2'-dimethyl-4,4'-bipyridylium dibromide (E_o' = -520 mV,n=1) and the N,N'-dimethyl-3-methyl -4,4'-bipridylium dibromide (E_o' = -617 mV,n=1); the latter two were gifts of Dr. B. White, I.C.I. Plant Protection Ltd., Bracknell, Berks. U.K.

RESULTS AND DISCUSSION

When native reduced HIPIP solution was mixed with DMSO, no changes were observed in its absorption spectrum until the DMSO concentration exceeded 70% by volume, when there was a shift in the absorption peak from 388 nm to 406 nm (Figure I). It seems likely that this change is due to a conformational

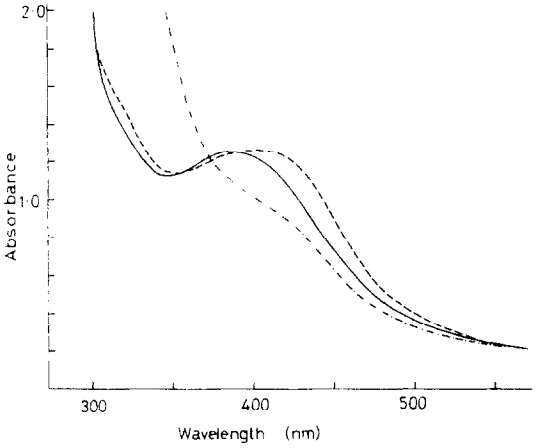

Fig. I. Optical absorption spectra of HIPIP, —— in 20 mM Tris-Cl, pH 9.0; --- in 80% DMSO; -·-·- in 80% DMSO, 5 min. after adding 0.25 mM sodium dithionite.

change induced in the protein, which is analogous to the changed induced in ferredoxins by a variety of denaturing agents (see reference 7). No EPR signals were detected from this form.

Provided the solution was kept anaerobic, the protein appeared to be stable in high concentrations of DMSO, and the normal absorption spectrum was restored by diluting the solution with anaerobic buffer solution until the DMSO concentration was below 70%. However, if oxygen was admitted, absorption bands appeared at 410 and 460 nm and the protein then underwent irreversible denaturation over the course of a few minutes.

On adding sodium dithionite solution to reduced HIPIP in 80% DMSO, there was a decrease in optical absorption similar to that observed on reduction of bacterial ferredoxin (Figure I). The dithionite reduced form showed an EPR signal at g = 2.04, 1.93 (Fig.2). Like the signals of reduced bacterial ferredoxins, the signal was observed only at temperatures below 35°K. The lineshape of this signal is similar to the reduced four-iron ferredoxin from Bacillus polymyxa (8), although the high-field g-values are less clearly separated. The decrease in A_{406}, and size of the EPR signal, were maximal when the HIPIP solution was adjusted to pH 9-10 before addition of DMSO.

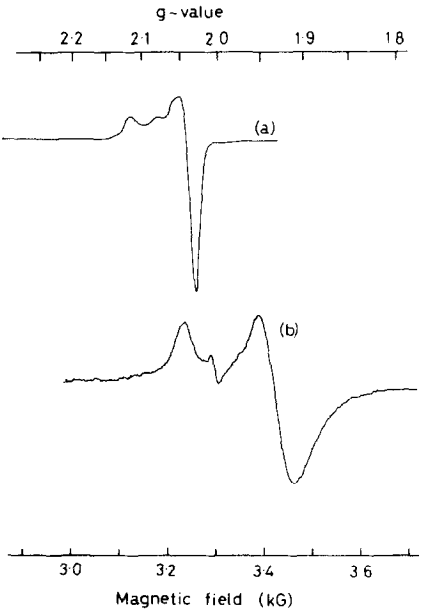

Fig. 2. EPR spectra of (a) 0.1 mM HIPIP, pH 9.0 oxidized with 1 mM ferricyanide; (b) 0.1 mM HIPIP in 80% DMSO, reduced with 1mM dithionite. For the latter spectrum the protein in an EPR tube was mixed with DMSO and kept for 3 min., then dithionite solution was added and after a further 3 min. the sample was frozen; all manipulations were carried out at 0°C under anaerobic conditions. The gain setting for Fig. 2 (b) is 20 x that for 2 (a). Other instrument settings for both spectra were: temperature, 18 K; microwave power 1 mW; microwave frequency 9.23 GHz; modulation amplitude 10 G.

The reduction could be reversed by addition of ferricyanide and repeated several times without loss of optical absorption or EPR signal intensity (see Fig.3). Moreover it was possible to reverse the treatment by reoxidizing the HIPIP with ferricyanide, then removing the DMSO by dilution in Tris-Cl buffer, followed by reconcentration on a small column of DEAE-cellulose.[7]. The recovery of intact HIPIP, as judged by the size of the EPR signal of the oxidized form (Fig. 2) was 77%. It therefore seems likely that during reduction in 80% DMSO, the four-iron centre of HIPIP remains intact. It is of interest that McDonald and co-workers (9) who observed the denaturation of C. pasteurianum ferredoxin in DMSO, using proton magnetic resonance, reported that under conditions where the protein was substantially unfolded, the four-iron clusters appeared to remain intact.

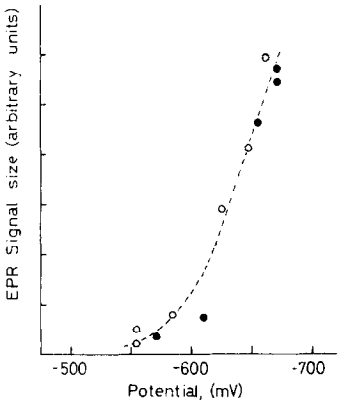

Fig. 3. Potentiometric titration of 0.05 mM HIPIP in 20 mM Tris-Cl, pH 9.0 + 80% DMSO. The titration was carried out in a vessel maintained at 0°C, bubbled with O_2 - free N_2, and the potential measured with platinum and calomel electrodes. Aliquots of sodium dithionite were added to reduce the protein, and samples were taken into matched EPR tubes flushed with N_2, and frozen. Points ● represent samples from the first reduction with dithionite. The protein was then reoxidized with potassium ferricyanide to a potential of -338 mV and points O were taken during a second course of reduction.

The EPR signal observed is unlikely to be due to contaminant ferredoxin, as the HIPIP used was established to be pure by several criteria. Moreover, no EPR signals were observed when the HIPIP, in the absence of DMSO, was treated with high concentrations of dithionite at any pH value between 5.5 and 12.5, even when low-potential redox mediators such as methyl viologen were added.

These results strongly suggest that in the presence of 80% DMSO HIPIP can be "super-reduced" to a state $C^{=}$, similar to reduced ferredoxin.

The magnitude of the EPR signal of the super-reduced form in Fig. 2(b) was estimated by numerical double integration, to represent 41% of that of oxidized HIPIP in Fig. 2 (a). Even allowing for the difficulties inherent in such comparisons, this discrepancy requires some explanation. It is possible that in 80% DMSO only a proportion of the HIPIP molecules are in a conformation suitable for super-reduction. However, no enhancement in signal size was observed on increasing the DMSO concentration to 95%. A more likely reason lies in the very low potential required for the reduction. Fig. 3 shows the results of a potentiometric titration of HIPIP in 80% DMSO. The shape of the graph of EPR signal size

against redox potential suggests that at the lowest potential which could be reached using dithionite, the magnitude of the EPR signal had not reached a maximum. These data indicate that the mid-point potential of the reduction is -640 mV or lower. This value, obtained under non-standard conditions is not directly comparable with redox potentials of ferredoxins because of the influence of DMSO on electrode junction potentials and pH, but it indicates that the reduction potential is in a similar range to those of the ferredoxins.

The super-reduction of HIPIP was only observed at DMSO concentrations high enough to shift the absorption maximum to 406 nm (Fig. I), suggesting that the protein must first undergo some conformational change. It should be possible, by decreasing the DMSO concentration around super-reduced HIPIP, to re-form the native protein conformation around the super-reduced four-iron centre. When this was done, by diluting with anaerobic buffer to a final concentration of 5% DMSO, there was a change in the EPR spectrum, as indicated by narrower linewidths and slightly increased g-values, but this state was unstable, and spontaneously reoxidized within a few minutes at 0°C, even in the presence of an excess of sodium dithionite. The redox potential of this change was too low to measure. This result indicates that native HIPIP cannot normally become super-reduced because its potential is too low.

It is noteworthy that a number of iron-sulphur proteins have been proposed to undergo energy-dependent changes in redox potential during their reactions. An example of this is the nitrogenase system, where the addition of ATP causes the iron-sulphur centre of the Mo-Fe protein to be reduced, if the Fe-protein is present (see Ref. 10). Since ATP causes a change in the EPR spectrum of the Fe-protein, it is suggested that it induces a decrease in its redox potential. The observations described in this paper provide a simple model for the change in redox potential induced by modification of protein structure.

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