Photochemical Generation of the Dichloroacetate Radical by Reduced Ferredoxin

Richard Cammack,[a] Ruth Williams,[a] K. Krishna Rao[b] and Martyn C. R. Symons[c]
[a] *Division of Biomolecular Sciences, King's College, London W8 7AH, UK*
[b] *Division of Biosphere Sciences, King's College, London W8 7AH, UK*
[c] *Department of Chemistry, University of Leicester, Leicester LE1 7RH, UK*

Solutions of reduced [2Fe–2S] ferredoxin from the cyanobacterium *Spirulina platensis*, are found to be sensitive to visible light in the frozen state at 77 K, if trichloroacetate is also present. An EPR-detectable radical is generated, which is stable for several hours at 77 K. The process is dependent on the presence of the protein and a reductant (dithionite). The shape of the radical spectrum is the same when the solvent is D_2O. It has been identified as the dichloroacetate radical anion, $^{\bullet}CCl_2CO_2^-$. The ferredoxin becomes partially oxidized in the photochemical process, in parallel with the appearance of the radical. On thawing the samples, the EPR spectrum of the reduced ferredoxin increases as the protein is partially re-reduced by dithionite. This is an unusual situation in which photochemically-induced electron transfer can be observed from a metal site within a protein.

Trichloroacetate anion is a 'chaotropic agent' which, at high concentrations, has the effect of decreasing the hydrophobic interactions within proteins. Chaotropic agents are used for partial denaturation of proteins, and extraction of membrane-bound proteins.[1] Trichloroacetate has been shown to cause changes in the conformation of iron–sulphur proteins, including plant-type ferredoxin, a protein containing a [2Fe–2S] cluster.[2] At high concentrations (*ca.* 2 mol dm^{-3} trichloroacetate), the ferredoxin is substantially unfolded, but at lower concentrations (100 mmol dm^{-3}), it undergoes a change in its EPR properties which indicates a subtle change in the structure of the iron–sulfur cluster.[1]

Trichloroacetate is also capable of forming a radical species by reductive elimination of chloride ion.[3] In this paper we describe a photochemical reaction between the reduced ferredoxin and trichloroacetate ion.

Experimental

Ferredoxins from *Spirulina platensis*, *S. maxima* and spinach were prepared as described by Hall *et al.*[4] Samples were made up in quartz tubes of 3 mm internal diameter, in 20 mmol dm^{-3} phosphate buffer, pH 7.0. They were illuminated at 77 K under liquid nitrogen, with a 300 W quartz-halogen spot lamp at a distance of 10 cm. Continuous-wave EPR measurements were made on a Bruker ESP300 spectrometer with an Oxford Instruments ESR900 flow cryostat.

The spectrum of the dichloroacetate radical was obtained by gamma-irradiation of a sample of trichloroacetate. The solvent system (CD_3OD–D_2O, 9:1) was selected because this is, at 77 K, a good medium for electron addition to solutes. On exposure to γ-rays at 77 K electrons are ejected almost entirely from solute molecules, but are not readily captured by the solvent.

Results

Fig. 1(*a*) shows EPR spectra of reduced *S. platensis* ferredoxin, in the presence of 90 mmol dm^{-3} trichloroacetate. On illumination with visible light in the frozen state, a radical was induced [Fig. 1(*b*)]. The signal was stable for several hours in the frozen state, but decayed rapidly when the sample was thawed [Fig. 1(*c*)]. Similar results were obtained with ferredoxins from the related organism *Spirulina maxima* and from spinach.

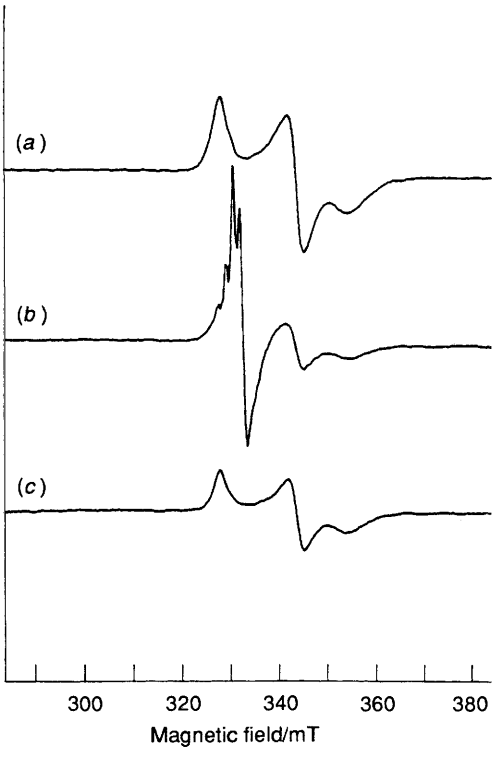

Fig. 1 (*a*) EPR spectrum of reduced *S. platensis* ferredoxin + trichloroacetate; (*b*) after 25 min illumination; (*c*) after thawing anaerobically and re-freezing. Conditions of measurement: temperature, 50 K; microwave power 20 mW; microwave frequency 9.338 GHz; modulation amplitude 0.16 mT.

Spectra of the radical, measured under non-saturating conditions, are shown in Fig. 2. Spectra of samples in H_2O and D_2O were identical, indicating that the hyperfine splittings in the radical spectrum are not due to exchangeable protons.

The most likely assignment of the radical signal is a dichloroacetate anion, formed by electron capture and the elimination of Cl^-. A spectrum of the $Cl_2\dot{C}CO_2^-$ radical is shown in Fig. 3. The electron loss centres ($CD_3\dot{O}D^+$ and $D_2O^{\bullet+}$) are both readily converted into $^{\bullet}CD_2OD$ radicals,

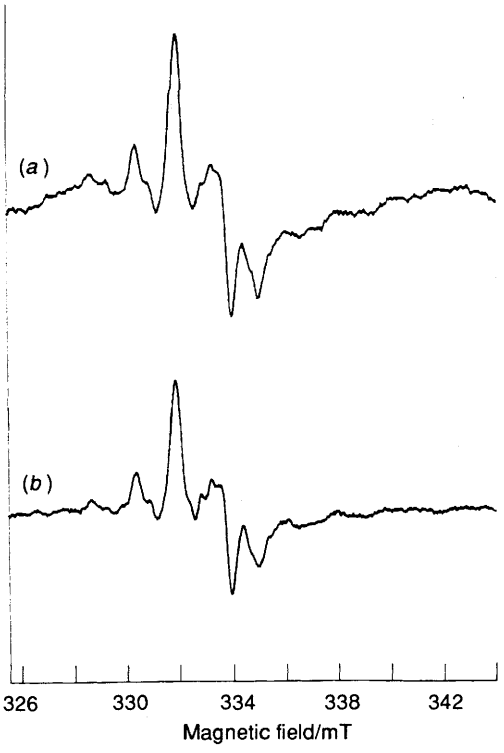

Fig. 2 EPR spectrum of radical induced by illumination of reduced ferredoxin in the presence of 89 mmol dm^{-3} trichloroacetate; (*a*) in H_2O; (*b*) in D_2O. Conditions of measurement: temperature, 50 K; microwave power 0.02 mW; microwave frequency 9.381 GHz; modulation amplitude 1.6 mT.

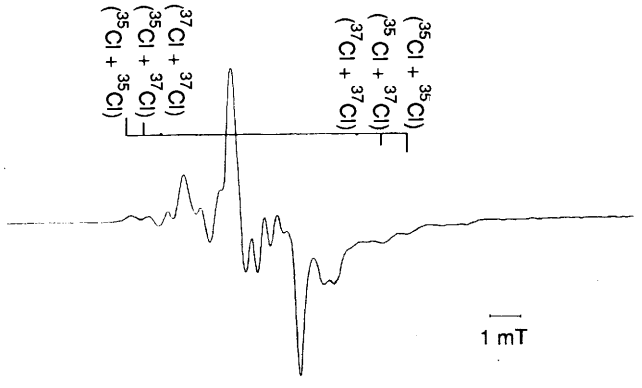

Fig. 3 EPR spectrum for $CCl_3CO_2^-Na^+$ in CD_3OD–D_2O, after exposure to ^{60}Co γ-rays at 77 K, and annealing to remove most of the solvent radicals. Conditions of measurement: temperature, 77 K; microwave power 1 mW; microwave frequency 9.228 GHz; modulation amplitude 0.5 mT. The stick diagram shows the outer lines of the first-order parallel features for (^{35}Cl + ^{35}Cl), (^{35}Cl + ^{37}Cl) and (^{37}Cl + ^{37}Cl) centres. There is a small quadrupole effect, which has not been allowed for.

Table 1 Parameters used in the simulation of the spectrum of $^{35}Cl_2CCO_2^-$

g_x	2.009
g_y	2.014
g_z	2.002
g_{av}	2.0083
A_x	−4.0
A_y	−4.0
A_z	21.0
A_{iso}	4.3

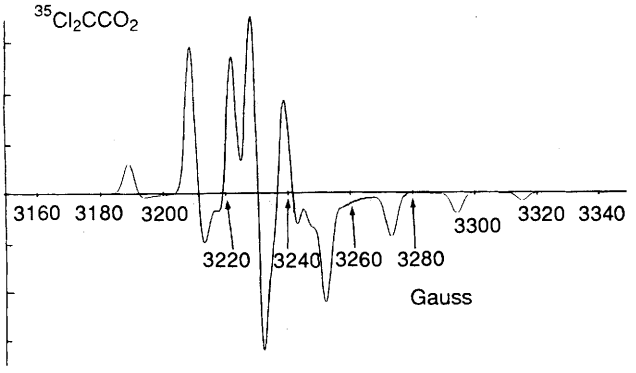

Fig. 4 Simulation for the (^{35}Cl + ^{35}Cl) species using the data given in Table 1

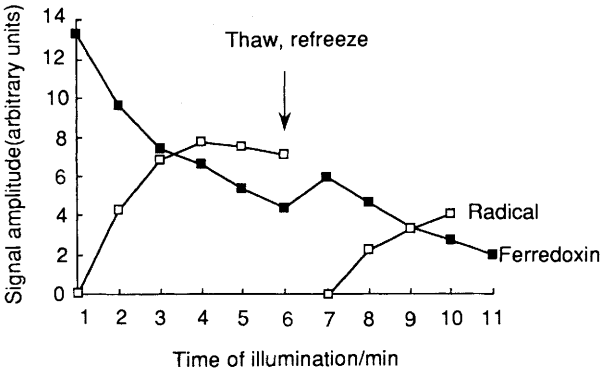

Fig. 5 Amplitudes of the EPR spectra of the iron–sulfur (■) and radical (□) species, as a function of time of illumination. At the time indicated by the arrow, the sample was thawed, to allow reduction of the ferredoxin by excess dithionite, and re-frozen. Spectra were recorded on the same sample at 50 K, using microwave power 20 mW for the ferredoxin and 0.02 mW for the radical signals.

which give a central quintet. Careful annealing has largely removed these features in the spectrum.

The radicals are thought to be planar, or nearly so, at the radical centre, and hence the *g*- and separate *A*-tensors are nearly coaxial. The $^{\bullet}CCl_3$ radical does not deviate far from planarity. Replacing –Cl by $-CO_2^-$ should strongly favour planarity because of improved π-overlap.[5,6]

A simulation of the radical spectrum, based on two ^{35}Cl nuclei, is shown in Fig. 4. Although the true spectrum will be more complex owing to the presence of ^{37}Cl nuclei, it can be seen that the general features of the radical spectrum are reproduced. The values used for the simulation are given in Table 1. The values agree well with the liquid phase data: g_{av} = 2.0083, A_{iso} = 0.31 mT.[7] The molecule is expected to be planar, with electron density distributed in the π-bonding system, so *g* and *A* are expected to be colinear.

Measurement of the EPR spectra of the radical species and reduced ferredoxin showed that the ferredoxin signal diminished in parallel with the appearance of the radical (Fig. 5). When the signals were measured under non-saturating conditions and integrated, the decrease in the amplitude of the iron–sulphur cluster signal and the appearance of the radical signal were in the ratio 1.06:1. When the sample was thawed and refrozen the spectrum of the reduced [2Fe–2S] cluster was partly restored (Fig. 5), consistent with re-reduction of part of the protein by the excess dithionite present. This result indicates that the ferredoxin was the electron donor involved in formation of the dichloroacetate radical, although some of the iron–sulphur clusters were modified, probably damaged, by the light treatment.

In control experiments (Table 2) it was shown that the radical

Table 2 Radical EPR signal amplitudes under different conditions. Signal sizes are relative.

Conditions	Radical signal amplitude
Complete[a]	100
Without trichloroacetate	<2
Without ferredoxin	2
Without dithionite	<2
Light $\lambda > 450$ nm[b]	5
Complete + 286 mmol dm^{-3} KCl	76

[a] Complete system: 0.28 mmol dm^{-3} ferredoxin, 2 mmol dm^{-3} dithionite, 89 mmol dm^{-3} trichloroacetate, 20 mmol dm^{-3} potassium phosphate, pH 7.0, illuminated for 30 min. [b] Light passed through a water heat filter and a Yellow No. 101 filter (Lee filters, Andover, England).

signal was not observed if either dithionite or trichloroacetate was omitted. In the absence of ferredoxin, only a very small radical signal was observed after illumination of a sample of phosphate buffer + dithionite + trichloroacetate, and this did not have the same line shape as Fig. 2. This indicates that it is the iron–sulfur cluster which is acting as the chromophore. Iron–sulfur clusters show absorption across the whole visible region. However the yield of radical was very small in samples illuminated through a filter transmitting mainly light wavelengths greater than 450 nm, from which it appears that blue and near-ultraviolet radiation is most effective.

The trichloroacetate ions which are involved in the reaction might be either bound to the protein or in solution. The changes in the EPR spectrum of plant-type ferredoxins were found to be prevented by the presence of high concentrations of salts. In the present study it was found that the photo-induced radical signal was somewhat diminished by the presence of 286 mmol dm^{-3} KCl (Table 2). This suggests there is some ionic contribution to the interaction of the protein with trichloroacetate.

Conclusions

The iron–sulfur cluster in ferredoxin is buried inside a hydrophobic region of the protein, though the iron atoms are within 0.5 nm of the surface and two of the cysteine sulfur ligands are accessible.[8,9] Electron-transfer reactions of ferredoxin in solution have been demonstrated with inorganic complexes.[10] Electron transfer in the present study occurred in the frozen state, to trichloroacetate molecules that were possibly loosely associated with the protein. A number of studies have shown the process of photochemically-induced electron transfer into metal centres within proteins, from agents such as ruthenium at defined sites on the protein.[10] These studies have shown that, depending on the intervening protein structure, electron transfer can take place over distances up to 2.0 nm.[11] In the present case the electron transfer is in the opposite direction, from the metal centre into the trichloroacetate ion, which acts as an electron trap. If chlorinated molecules can be specifically located on the protein, the phenomenon may have further applications for the study of long-range electron transfer.

Acknowledgements

This work was supported by grants from the UK Science and Engineering Research Council to R. C. We thank Mr. A. C. White for technical assistance.

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Paper 2/01785G
Received 30*th March* 1992
Accepted 9*th April* 1992