Biochimica et Biophysica Acta 932 (1988) 124–129
Elsevier

BBA 42693

A study of the reaction between spinach ferredoxin and one-electron reduced herbicides of differing charge

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(Received 5 October 1987)

Key words: Ferredoxin; Methyl viologen; Pulse radiolysis; Electrochemistry; Ionic strength; (Spinach)

The electron-transfer reactions involving spinach ferredoxin and two Photosystem I herbicides, methyl viologen and 2,1,3-benzothiadiazole-4,7-dicarbonitrile (BTDN) with similar one-electron reduction potentials (−446 mV and −490 mV, respectively, at pH 7), but with differing charges, have been investigated in aqueous solution by pulse radiolysis. In the one-electron reduced state, methyl viologen has one positive charge and BTDN has one negative charge, whereas ferredoxin has a calculated net negative charge of −16 at pH 7. Their reactions with ferredoxin appear to proceed via simple second-order electron-transfer reactions without complex formation. The rate constant for the reaction between methyl viologen monocation radical and ferredoxin at pH 7.0 and an ionic strength of 0.1 M is $8.9 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ and, for the reverse reaction, $4.0 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. The reaction with BTDN was about 10-fold slower but both BTDN and methyl viologen react considerably more rapidly with ferredoxin than either O_2 or H_2O_2 (Hosein, B. and Palmer, G. (1983) Biochim. Biophys. Acta 723, 383–390), even though the latter reactions are considerably more thermodynamically favourable. Increasing ionic strength (from 0.1 M to 0.5 M) decreased the observed first-order rate constant for the reaction of reduced methyl viologen with ferredoxin, but increased that for BTDN, as predicted from the charges on the reaction partners.

Introduction

Spinach ferredoxin is a small hydrophilic protein with a binuclear iron-sulphur cluster which functions as the natural electron acceptor for electrons from the membrane-bound iron-sulphur clusters Fe-S_A and Fe-S_B of chloroplast Photosystem I [1–4]. Reduced ferredoxin is normally re-oxidised by $NADP^+$ in a reaction catalysed by the flavoprotein ferredoxin-$NADP^+$ oxidoreductase [3,5], but when $NADP^+$ is limiting, ferredoxin can react with oxygen, superoxide and hydrogen peroxide [6,7].

A detailed kinetic study of the reactions of reduced ferredoxin with oxygen and hydrogen peroxide has been performed by Hosein and Palmer [7]. They showed that ferredoxin reacts with hydrogen peroxide in a second-order reaction with a rate constant of $5.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, and that the

Abbreviations: MV^{2+}, oxidised methyl viologen; MV^+ methyl viologen radical; E_7^1, one-electron reduction potential at pH 7; BTDN, 2,1,3-benzothiadiazole-4,7-dicarbonitrile; $BTDN^-$, reduced BTDN; fd, ferredoxin; μ, ionic strength.

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reaction with oxygen proceeds at a rate 10-fold slower and involves complex formation between the ferredoxin and oxygen. They suggested that these reactions may be involved in removing toxic oxygen species generated in chloroplasts.

The level of hydrogen peroxide in chloroplasts is increased in the presence of the herbicide methyl viologen (paraquat, MV^{2+}). This is reduced by Photosystem I in competition with ferredoxin and the resulting radical is re-oxidised by oxygen (rate constant $7.7 \cdot 10^8$ $M^{-1} \cdot s^{-1}$) generating superoxide [4,9–12]; the dismutation of this superoxide to form hydrogen peroxide is accelerated by chloroplastic superoxide dismutase.

The rates of reaction of reduced ferredoxin with O_2 and H_2O_2 are relatively slow. In this study, we have used pulse radiolysis to determine the rate constants for the electron transfer reactions involving methyl viologen and ferredoxin in order to compare the rates with those for the reactions of ferredoxin with oxygen and hydrogen peroxide.

Ferredoxin carries a large negative charge at neutral pH, calculated from the amino acid sequence [13] to be -16 assuming the single histidine residue is unprotonated and the oxidised iron-sulphur cluster has a net charge of -2 [14]. Since oxidised methyl viologen carries a double positive charge, a favourable electrostatic interaction between reduced ferredoxin and oxidised methyl viologen would influence the reaction between them. We have therefore also studied the effects of ionic strength on the reactions between ferredoxin and both methyl viologen and another Photosystem I herbicide, 2,1,3-benzothiadiazole-4,7-dicarbonitrile (BTDN), which is neutral in the oxidised state but has a similar one-electron reduction potential (-490 mV at pH 7) to that of methyl viologen (-446 mV at pH 7) [15,16].

The results show that although the electrostatic interaction between methyl viologen and ferredoxin accelerates the reaction rate, both methyl viologen and BTDN react considerably more rapidly with ferredoxin than either O_2 or H_2O_2, even though the latter reactions are very much more thermodynamically favourable. The significance of these results to any role of ferredoxin in removing toxic oxygen species in plants treated with Photosystem I herbicides is discussed.

Experimental

The 4.3 MeV linear accelerator and the optical detection and data storage systems used for pulse radiolysis studies have been described [17] previously. All solutions were prepared using water which had been purified using a Millipore Milli-Q system. Solutions were buffered at pH 7.0 ± 0.2 using 5 mM phosphate and contained 0.1 M sodium formate in order to convert hydroxyl ($^{\cdot}OH$) radicals and hydrogen atoms ($^{\cdot}H$) into $CO_2^{\cdot-}$ as shown in reactions (1) and (2).

$$H_2O \rightsquigarrow {}^{\cdot}OH, {}^{\cdot}H, e_{aq}^- \quad (1)$$

$$({}^{\cdot}H) {}^{\cdot}OH + HCO_2^- \rightarrow (H_2)H_2O + CO_2^{\cdot-} \quad (2)$$

The presence of e_{aq}^- and $CO_2^{\cdot-}$ makes the system wholly reducing. Prior to irradiation the solutions were deoxygenated by purging with N_2 (Air Products, high purity grade) for 20 min followed by a further 5 min after addition of ferredoxin to the solution. The deoxygenated solutions contained in a quartz cell of 0.7 cm pathlength were irradiated at 294 ± 3 K with electron pulses of 1.6 μs duration. Radiation doses of 4–5 Gy/pulse were determined using KSCN dosimetry at 480 nm assuming $G = 0.3$ μmol $\cdot$ J^{-1} and $\epsilon = 710$ $m^2 \cdot mol^{-1}$.

The one-electron reduction potential of ferredoxin was determined using the methods previously described [18].

MV^{2+} was obtained from Aldrich and BTDN was provided by the Biological Sciences Research Centre, Shell Development Company, Modesto, CA. All other chemicals were of Analar grade and used as supplied. Ferredoxin (fd) was extracted and purified from spinach leaves essentially according to the method of Rao et al. [19] except that further purification was achieved by gel filtration on a Sephadex G50 (superfine) column rather than by hydroxyapatite chromatography. The ferredoxin was eluted using 20 mM potassium phosphate buffer pH 7.5 and stored in liquid nitrogen until required. The gel filtration increased the purity of the ferredoxin as well as separating it from the NaCl added earlier. Ferredoxin concentrations were determined spectrophotometrically using an extinction coefficient at 420 nm of 940 $m^2 \cdot mol^{-1}$. The ferredoxin obtained from the final purification step had an absorption ratio

($A_{420\,nm}/A_{275\,nm}$) of greater than or equal to 0.43 (pure ferredoxin has a ratio of 0.48).

Results

Interaction of MV^+ with ferredoxin

On pulse irradiation of deoxygenated, aqueous solutions containing MV^{2+} (16–27 μM) and ferredoxin (6–15 μM) and buffered at pH 7.0, optical absorption changes with time were measured at 600 nm and 460 nm and typical results are shown in Fig. 1. The optical absorption change at 600 nm is due to the formation of the one-electron reduced species of MV^{2+}, MV^+ [12], which in the absence of ferredoxin is stable on the timescale shown in Fig. 1. At 600 nm, after initial formation of MV^+ via reactions (3) and (4) a subsequent partial loss of MV^+ was observed in the presence of ferredoxin. After approx. 1.5 ms, an equilibrium situation is attained (reaction 5).

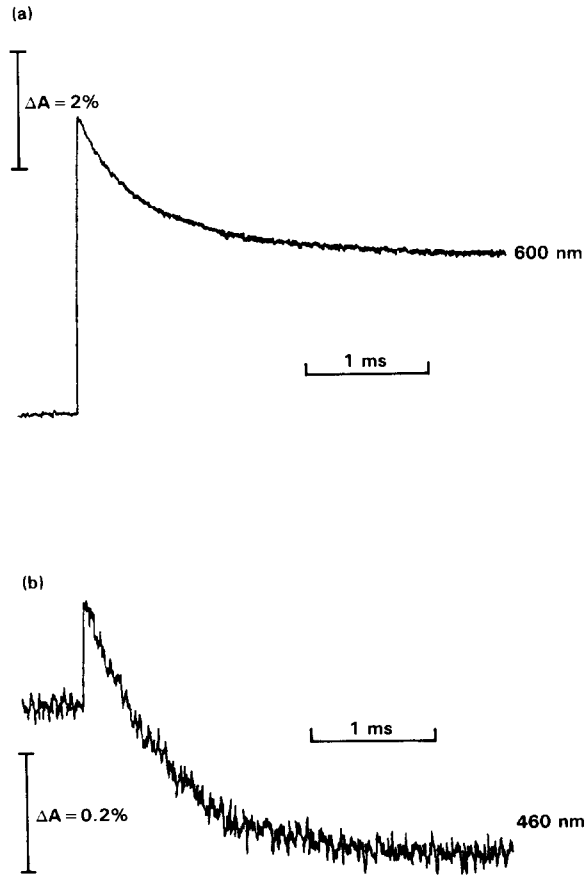

Fig. 1. Absorption changes with time determined at 600 nm (a) and 460 nm (b) upon pulse irradiation of deoxygenated, aqueous solutions containing 18.3 μM MV^{2+}, 6.6 μM ferredoxin and 0.1 M formate at pH 7.

$$e_{aq}^- + MV^{2+} \rightarrow MV^+ \quad (3)$$

$$CO_2^- + MV^{2+} \rightarrow MV^+ + CO_2 \quad (4)$$

The time-scale of these changes at 600 nm parallel those at 460 nm, a wavelength at which reduced ferredoxin absorbs less strongly than the oxidised form [20]. After an initial increase in absorption due to the formation of MV^+ a subsequent 'bleaching' of absorption was observed at 460 nm due to the reduction of ferredoxin and the concomitant loss of MV^+. These changes with time at both 600 nm and 460 nm are consistent with the establishment of equilibrium (Eqn. 5), where fd and fd′ are the oxidised and reduced forms of ferredoxin, respectively.

$$MV^{2+} + fd' \underset{k_b}{\overset{k_f}{\rightleftharpoons}} MV^+ + fd \quad (5)$$

The establishment of equilibrium (Eqn. 5) is substantiated by the fact that the concentration of reduced ferredoxin corresponds with the concentration of MV^+ removed within approx. 1.5 ms (see Fig. 1) assuming $\epsilon_{600}^{MV^+} = 1370\ m^2 \cdot mol^{-1}$ [21] and ${}^{fd}\Delta\epsilon_{460} = 440\ m^2 \cdot mol^{-1}$ [20] and taking into account the residual absorption of MV^+ at 460 nm at equilibrium. On the longer time-scale there is no obvious change in absorption which would correspond to a previously reported change using CO_2^- as reducing agent [22]. This lack of any further change in absorption is consistent with the observations of Adzamli et al. [20].

The kinetics of the rate of change of optical absorption with time at both 460 nm and 600 nm is first order. Within experimental error, the rate constants determined at both wavelengths are equivalent. Assuming the establishment of equilibrium (Eqn. 5), the dependence of the determined first-order rate constants (k_{obs}) for various concentration ratios of [MV^{2+}]:[ferredoxin] as represented by Eqn. 6 is shown in Fig. 2.

$$k_{obs} = k_f[MV^{2+}] + k_b[fd] \quad (6)$$

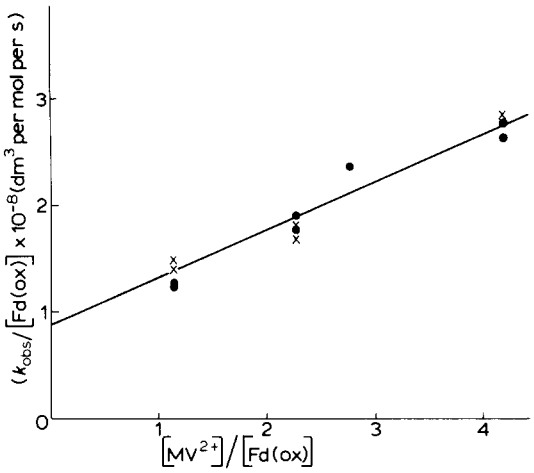

Fig. 2. The dependence of the rate of approach to equilibrium upon the concentration ratio $[MV^{2+}]$:[ferredoxin] as determined at 600 nm (●) and 460 nm (×).

From this dependence, the values for k_b and k_f were determined to be $8.9 \cdot 10^7$ and $4.0 \cdot 10^7$ $M^{-1} \cdot s^{-1}$, respectively. From the ratio of k_f to k_b the one-electron reduction potential (E_7^1) of fd was estimated to be -426 ± 15 mV (at room temperature) based upon Eqn. 7 and taking the E_7^1 value of MV^{2+} to be -446 ± 15 mV. The E_7^1 value of ferredoxin is in good agreement with that calculated from the position of equilibrium (see later) and the value determined electrochemically [1,2].

$$E = E_7^{1(MV^{2+}/MV^+)} - E_7^{1(fd/fd')} = -\frac{RT}{nF} \ln K \approx 59 \log \frac{k_f}{k_b} \quad (7)$$

The E_7^1 value of ferredoxin was also determined from the concentrations of MV^+ and reduced ferredoxin after establishment of equilibrium (5). Taking the E_7^1 value of MV^{2+} to be -446 mV the E_7^1 value of ferredoxin was determined to be -430 ± 10 mV and the equilibrium constant for (Eqn. 5) as 0.5 ± 0.1.

Effect of ionic strength

In order to investigate the effect of ionic strength μ, the rate of change of optical absorption with time was determined at both 600 and 460 nm on pulse irradiation of aqueous solutions containing MV^{2+} (18–18.5 μM), ferredoxin (6.6

TABLE I

THE EFFECT OF IONIC STRENGTH ON THE E_7^1 OF FERREDOXIN AND THE RATE CONSTANTS FOR THE INTERACTION OF FERREDOXIN WITH MV^+ OR $BTDN^-$

The $-E_7^1$ values are not corrected for changes in activity coefficients at high ionic strength. n.d., not determined due to slow kinetics.

Substrate	[NaCl] (M)	μ (M)	$-E_7^1$ (mV)	$10^{-3} \cdot k_{obs}$ (s^{-1})	
				600 nm	460 nm
MV^{2+}	0	0.1	430	1.6	1.7
	0.18	0.28	424	0.6	0.5
	0.4	0.5	≈ 396	0.5	0.5
BTDN	0	0.1	n.d.	≈ 0.2	≈ 0.18
	0.4	0.5	≈ 440	0.3	0.3

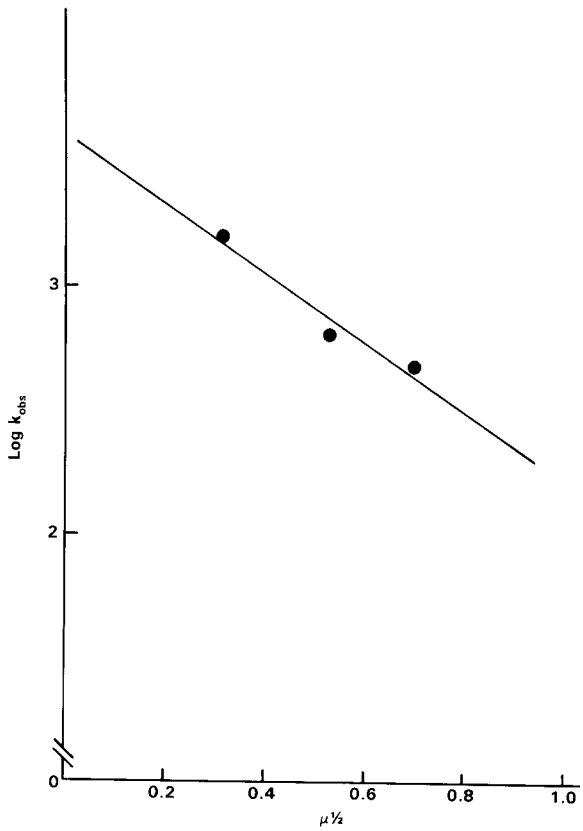

Fig. 3. The dependence of the rate constant for approach to equilibrium upon ionic strength determined on pulse irradiation of deoxygenated solutions containing 18.3 μM ferredoxin, 0.1 M formate and 0–0.4 M chloride ion at pH 7.

μM), formate (0.1 M) and sodium chloride (0.18–0.4 M) at pH 7. The determined first-order rate constants for the approach to equilibrium (5) and the E_7^1 values of ferredoxin calculated from the equilibrium concentrations are presented in Table I for different ionic strengths.

The E_7^1 values are essentially independent of μ and have not been corrected for changes in activity coefficients at high ionic strength. In calculating the values of E_7^1 for ferredoxin, it was assumed that the E_7^1 value for methyl viologen is independent of ionic strength. The $E_{1/2}$ value of methyl viologen as determined by polarography in 0.01 M phosphate buffer, pH 7.0, varied by not more than 12 mV when the ionic strength was varied from 0.1 to 0.6 M by the addition of NaCl (result not shown). The E_7^1 value of ferredoxin at 0.4 M NaCl is only approximate, since the establishment of the equilibrium is slow and the quality of the absorbance-time profiles decreased due to lamp instability. The other important observation is that the rate constants for approach to equilibrium decrease with μ. The dependence of the rate constant upon $\mu^{1/2}$ is shown in Fig. 3 with a slope of about -1.4. Since a macromolecule is involved and μ is high, meaningful parameters based upon the Debye-Huckel relationship are not obtainable.

Interaction of one-electron reduced species of BTDN with ferredoxin

The interaction of one-electron reduced BTDN ($E_7^1 = -490$ mV) [16] with ferredoxin was followed after pulse irradiation of aqueous solutions containing BTDN (22.2 μM), ferredoxin (6.7 μM) and formate (0.1 M) buffered at pH 7. The optical absorption changes were monitored at 680 nm, a wavelength at which the one-electron reduced species of BTDN absorbs with a molar extinction coefficient of 300 $m^2 \cdot mol^{-1}$ [16]. The rate constants for the reaction were determined in the absence and presence of 0.4 M NaCl and are presented in Table I. The rate constants in the absence of NaCl are about an order of magnitude less than that determined with methyl viologen. However, contrary to the observations with MV^{2+}, the rate constant increases (more than 30%) on increasing ionic strength and at 0.4 M NaCl is similar to that with MV^{2+}. It should be noted that the one-electron reduced species of BTDN has unit negative charge at pH 7 [16]. The E_7^1 value of ferredoxin estimated from the equilibrium with BTDN is in reasonable agreement with that determined using MV^{2+}.

Discussion

The reactions between ferredoxin and methyl viologen appear to proceed via simple second-order electron-transfer processes and the kinetic data give no indication of formation of transient intermediate complexes. The rate constants for the reactions ($8.9 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ for ferredoxin reduction and $4.0 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ for ferredoxin oxidation) by methyl viologen are, however, less than would be expected if diffusion-controlled.

The E_7^1 value of ferredoxin obtained from the equilibrium position with methyl viologen after pulse radiolysis is similar to that determined electrochemically [1,2]. Based upon the value determined, equilibrium (5) favours production of reduced ferredoxin under equi-concentration conditions of methyl viologen and ferredoxin. Since the redox potention of methyl viologen is essentially independent of ionic strength in the range studied, the redox potential of ferredoxin does not appear to be strongly influenced by ionic strength, confirming the observation of Stombaugh et al. [23]. Indeed the position of equilibrium (5), based upon the determined E_7^1 values at higher ionic strength, still favours formation of reduced ferredoxin.

The witnessed effects of ionic strength upon the rate constants for the interactions of ferredoxin studied (Table I) are consistent with the charges on methyl viologen and BTDN and with ferredoxin possessing an overall negative charge [13,24] or that the active site and its access are negatively charged. The structure of *Spirulina platensis* ferredoxin, as determined by X-ray crystallography [24] and which, based on sequence homology, is likely to be very similar to spinach ferredoxin, indicates that the iron-sulphur cluster is located near the molecular surface and is surrounded mainly by hydrophobic residues. Charged residues near the cluster are arginine 42 and glutamates 94, 95 and 96, probably giving this region a net negative charge. Whether the reduction in reactivity

with MV^+ is also indicative of a specific inhibitory effect on the ferredoxin by chloride ion is as yet not known. Even if a partial inhibition occurs, the rate constant increase with BTDN is consistent with the effect of ionic strength on the reactivity of anionic species.

The reactions of both methyl viologen and BTDN with ferredoxin are clearly considerably more rapid than those of either O_2 or H_2O_2. This would not be predicted on thermodynamic grounds alone (E_7^1 values for reduction of O_2 and H_2O_2 are -155 mV and $+300$ mV, respectively [25]). The effects of ionic strength on the reactions of methyl viologen and BTDN show that the overall charges on the molecules do affect the reaction rates but this cannot fully explain the large difference in reaction rates between O_2/H_2O_2 and the two low-potential compounds. Hosein and Palmer [7] showed that the reaction of O_2 with ferredoxin proceeds via complex formation, and this difference in reaction mechanism may provide an explanation for the sluggish kinetics in the case of O_2.

Our results show that in chloroplasts treated with methyl viologen, any electrons delivered to ferredoxin by Photosystem I are likely to reduce $NADP^+$ or MV^{2+}, rather than directly reducing O_2 or H_2O_2. Thus methyl viologen could stimulate O_2 reduction in chloroplasts by catalysing electron transfer to O_2 from iron sulphur centres FeS_A and FeS_B of Photosystem I, but also from ferredoxin. In the presence of methyl viologen any role for ferredoxin in removing hydrogen peroxide is more likely to be linked to $NADP^+$ reduction for reducing glutathione than in direct reduction of hydrogen peroxide.

Acknowledgements

C.M.T. is supported by a Research Assistantship from the Science and Engineering Research Council. We are indebted to Mr. T. Jenner for maintaining the radiation facility.

References

1 Tagawa, K. and Arnon, D.I. (1962) Nature 195, 537–543.
2 Hall, D.O. and Evans, M.C.W. (1969) Nature 223, 1342–1348.
3 Whatley, F.R., Tagawa, K. and Arnon, D.I. (1963) Proc. Natl. Acad. Sci. USA 49, 266–270.
4 Zweig, G., Shavit, N. and Avron, M. (1965) Biochim. Biophys. Acta 109, 332–346.
5 Maskiewicz, R. and Bielski, B.H.J. (1982) Biochim. Biophys. Acta 680, 297–303.
6 Allen, J.F. (1977) Curr. Adv. Plant Sci. 29, 459–469.
7 Hosein, B. and Palmer, G. (1983) Biochim. Biophys. Acta 723, 383–390.
8 Allen, J.F. (1975) Nature 256, 599–600.
9 Harbour, J.R. and Bolton, J.R. (1975) Biochim. Biophys. Res. Commun. 3, 803–807.
10 Chia, L.S., McRae, D.G. and Thompson, J.E. (1982) Physiol. Plant 56, 492–499.
11 Summers, L.A. (1980) in The Bipyridinium Herbicides, pp. 316–319, Academic Press, London.
12 Farrington, J.A., Ebert, M., Land, E.J. and Fletcher, K. (1973) Biochim. Biophys. Acta 314, 372–381.
13 Matsubara, H. and Sasaki, R.M. (1968) J. Biol. Chem. 243, 1732–1757.
14 Thomson, A.J. (1985) in Metalloproteins. Part 1. Metal Proteins with Redox Roles. (Harrison, P., ed.), pp. 79–120, Verlag Chemie GmbH, Weinheim.
15 Fielden, R. and Summers, L.A. (1974) Experientia 30, 843–844.
16 Camilleri, P., Dearing, A., Cole-Hamilton, D.J. and O'Neill, P. (1986) J. Chem. Soc. Perkin Trans II, 569–572.
17 O'Neill, P. and Chapman, P.W. (1985) Int. J. Radiat. Biol. 47, 71–80.
18 Wardman, P. and Clarke, E.D. (1976) J. Chem. Soc. Faraday Trans. I 72, 1377–1390.
19 Rao, K.K., Cammack, R., Hall, D.O. and Johnson, C.E. (1971) Biochem. J. 122, 257–265.
20 Adzamli, I.K., Kim, H.O.W., Sykes, A.G. and Buxton, G.V. (1982) J. Inorg. Biochem. 16, 311–317.
21 Farrington, J.A., Ebert, M. and Land, E.J. (1978) J. Chem. Soc. Faraday Trans. 1, 74, 665–675.
22 Maskiewicz, R. and Bielski, B.H.J. (1981) Biochim. Biophys. Acta 638, 153–160.
23 Stombaugh, N.A., Sundquist, J.E., Burns, R.H. and Orme-Johnson, W.H. (1976) Biochemistry 15, 2633–2641.
24 Fukuyama, K., Hase, T., Matsumoto, S., Tsukihara, T., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K. and Matsubara, H. (1980) Nature 286, 522–524.
25 Meisel, D. and Czapski, G. (1975) J. Phys. Chem. 79, 1503–1509.
26 Foyer, C.H. and Halliwell, B. (1976) Planta 133, 21–25.