Radiation-induced Electron Capture by Proteins containing Disulphide Linkages: an Electron Spin Resonance Study

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Exposure of dilute solutions of a wide range of proteins, enzymes, or polypeptides containing disulphide bonds to ^{60}Co γ-rays at 77 K gave readily detectable yields of $(RSSR)^-\sigma^*$ radicals, characterised by their e.s.r. spectra. The results suggest that electron capture at RSSR groups is remarkably selective, in the absence of other groups with higher electron affinities. In the absence of RSSR groups electron capture gave organic radicals (*g ca.* 2.002) presumably formed by dissociative electron capture at amide linkages. It is suggested that this method could be developed into a sensitive test for RSSR groups. These results imply that electrons added to proteins can move efficiently within the protein until they encounter S–S bonds. In the absence of such bonds and of other groups with high electron affinity they remain in the protein until they are trapped at amide linkages. In the particular case of the Cu^{II} form of superoxide dismutase, preferential electron capture occurred at Cu^{II}. However, after conversion to Cu^{I}, the expected $(RSSR)^-$ radicals were detected.

The radical chemistry of organic thiols, sulphides, and disulphides is of considerable complexity and importance, and radical intermediates have been extensively studied by optical [1-3] and e.s.r. [4-6] methods. It is now clear that well defined e.s.r. features originally assigned to RS· radicals are not due to such radicals.[7] We have suggested that the species concerned is an adduct of RS· and R_2S or RSH molecules [$RS^{\therefore}SR_2$] but Hadley and Gordy, who clearly established the presence of two non-equivalent sulphur atoms, prefer to assign the spectrum to RSS· radicals.[8] Because of this ambiguity this species is referred to X.[5] It seems possible that these ($RS^{\therefore}SR_2$ and RSS) species have similar e.s.r. spectra, since there are cogent reasons for accepting one or other structure under different conditions.[5,9] We have shown that RS· radicals, which are expected to have variable *g*-tensor components depending on their environment, can be characterised by their spectra in dilute methanolic solutions at 77 K.[5]

Disulphide radicals are of two types, $(RSSR)^+$ cations with a π* structure, and $(RSSR)^-$ with a σ* structure.[5,6,8,10,11] These can be readily formed from RSSR molecules by the use of ionizing radiation, and they both have well defined, characteristic, *g*-tensors. Yet another important class of σ* radicals are the $(R_2S^{\therefore}SR_2)^+$ cations, formed by the addition of $R_2S^{\cdot+}$ cations (also characterised by e.s.r. spectroscopy [12]) to R_2S molecules.[5,6,13]

When simple RSSR molecules in dilute solution in such solvents as CD_3OD are exposed to ^{60}Co γ-rays at 77 K, electrons ejected from solvent molecules add efficiently, to give $(RSSR)^-$ σ* anions. These are characterised by their *g*-tensor components ($g_\perp$ *ca.* 2.020, $g_\parallel$ *ca.* 2.002) and often also by their proton hyperfine coupling constants (Figure 1). Although the features are broad, they are sufficiently distinctive to be taken as being characteristic of $(RSSR)^-$ radicals. Disulphides play a major structural role in certain enzymes or proteins, and one aim of this study was to examine the possibility that electrons might selectively add to these units during radiolysis of dilute solutions at 77 K. Since electrons react with amide units and other protein components under these conditions, it was by no means certain that such selectivity would be observed.

Our other major aim was related to this. Previous studies of certain metallo-proteins under these conditions have shown that electron-capture at metal centres can be surprisingly efficient.[14-16] We were interested in finding out if this apparent selectivity is peculiar to metal-ion centres such as iron, molybdenum, and copper, or if it is a general property of proteins and enzymes. If it is, it may be a matter of some importance to their chemistry and physics.[17]

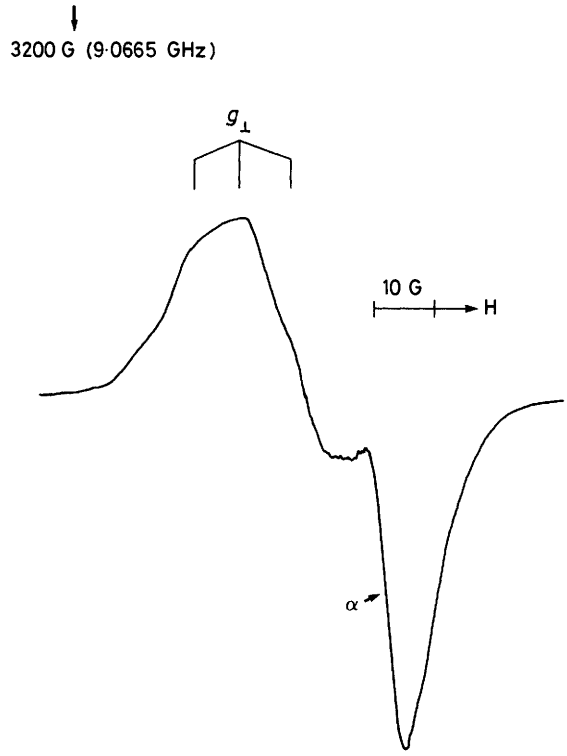


Figure 1. First derivative X-band e.s.r. spectrum for a dilute solution of cystine in D_2O + CD_3OD (4 : 1 v/v) after exposure to ^{60}Co γ-rays at 77 K and annealing to remove signals from solvent radicals, showing features assigned to cystine radical-anions, together with feature α assigned to carbon-centred radicals

Previous e.s.r. studies of sulphur radicals in some proteins include the pioneering work of Singh and Ormerod [18] and of Stratton.[19]

Experimental

The proteins, enzymes, and peptides used in this study, listed in the Table, were purchased from Sigma Chemicals and used

E.s.r. data for electron-addition products formed by radiolysis of dilute solutions of proteins and related compounds in D_2O–CD_3OD (4 : 1 v/v) glasses after annealing to *ca.* 180 K to remove signals from solvent radicals

Protein	Source	Proton hyperfine coupling (G) [a]	*g*-tensor components		
			g_x	g_y [c]	g_z
Alcohol dehydrogenase	Yeast	9.5	2.019	*ca.* 2.019	2.002
Glutathione (oxidised)	Synthesised	*b*	2.017	2.017	2.002
α-Chymotrypsin	Bovine pancreas	8.0	2.021	2.014	2.002
α-Lactolbumin	Bovine milk	9.5	2.020	*ca.* 2.020	2.002
Conalbumin	Hen's egg	*b*	2.020	*ca.* 2.020	2.002
Insulin	Bovine pancreas	9.0	2.020	*ca.* 2.020	2.002
Venom	Formosan cobra	7.5	2.020	*ca.* 2.020	2.002
Oxytocin	Synthetic	8.0	2.020	*ca.* 2.020	2.002
Lysozyme	Hen's egg	9.0	2.024	2.017	2.002
Glutathione reductase	Yeast	8.0	2.024	2.015	2.002
Lipoamide dehydrogenase	Porcine heart	8.0	2.024	2.015	2.002
Thyroglobulin	Bovine	*b*	*ca.* 2.018	*ca.* 2.018	*ca.* 2.002
Superoxide dismutase	Bovine	*b*	*ca.* 2.018	*ca.* 2.018	*ca.* 2.002
Albumin	Bovine serum	11.0	2.018	2.018	2.002
Ribonuclease	Bovine pancreas	9.0	2.019	2.019	2.002
Homocystine	Synthetic	8.5	2.019	2.019	*ca.* 2.002
Cystine	Synthetic	8.5	2.019	2.019	*ca.* 2.002
Cystine HCl [d]	Synthetic, crystal	10.3 + 7.2	2.0178	2.0174	2.0024

[a] G = 10^{-4} T: In all cases for which proton hyperfine coupling was resolved, a broad triplet similar to that for cystine anions was observed. [b] Very broad components: hyperfine coupling not resolved. [c] In most cases, g_z and g_y were not sufficiently defined for us to draw a clear distinction. [d] Ref. 6.

as supplied. Dilute solutions, generally in D_2O + CD_3OD (*ca.* 4 : 1 v/v) but sometimes also in water–glycol mixtures, were frozen as small glassy beads in liquid nitrogen and irradiated with ^{60}Co γ-rays at 77 K in a Vickrad source with doses up to *ca.* 1 Mrad.

E.s.r. spectra were measured at 77 K with a Varian E-109 X-band spectrometer calibrated with a Hewlett–Packard 5246L frequency counter and a Bruker BH 12E field probe, which were standardised with a sample of DPPH. Data were recorded after annealing to *ca.* 180 K to remove features from solvent radicals.

Results and Discussion

Our results are remarkably simple: for the very wide range of proteins and related compounds containing S–S linkages listed in the Table, good spectra were obtained for $(RSSR)^-$ σ* anions. In all cases, dilute aqueous glasses were used at 77 K, and hence the only major reaction expected for the proteins is electron addition. The efficient formation of these anions was independent of the site of the S–S linkages and of the number of such linkages in each molecule. The yields were linear with dose during *ca.* 1.0 h (1 Mrad), but tended to level off after this for solutions *ca.* 10^{-2} mol dm^{-3} in S–S linkages. The results are summarised in the Table, which also lists key references to the proteins involved. A typical e.s.r. spectrum is shown in Figure 2: the only variations were minor differences in g_x and g_y, and in the small additional yield of radicals with all *g* values close to 2.0023. In each case, it was possible to anneal the glasses to temperatures at which the [·OH] and [·CD_2OD] radicals fell to zero with no apparent loss in signals from protein radicals.

Proteins containing no S–S linkages appeared to add electrons with roughly the same efficiency, but we were unable to detect any clear structure in the broad features at *ca.* 2.0023 so we cannot identify these radicals from this spectra. The presence of RSH units made no difference and, in particular, there was no evidence for the formation of RS· radicals.[5]

It is remarkable that cystine and homocystine actually gave higher yields of carbon-centred radicals than the proteins–enzymes listed in the Table, as can be judged by comparing Figures 1 and 2. This probably reflects the fact that NH_3 is a good leaving group, and hence deamination competes with

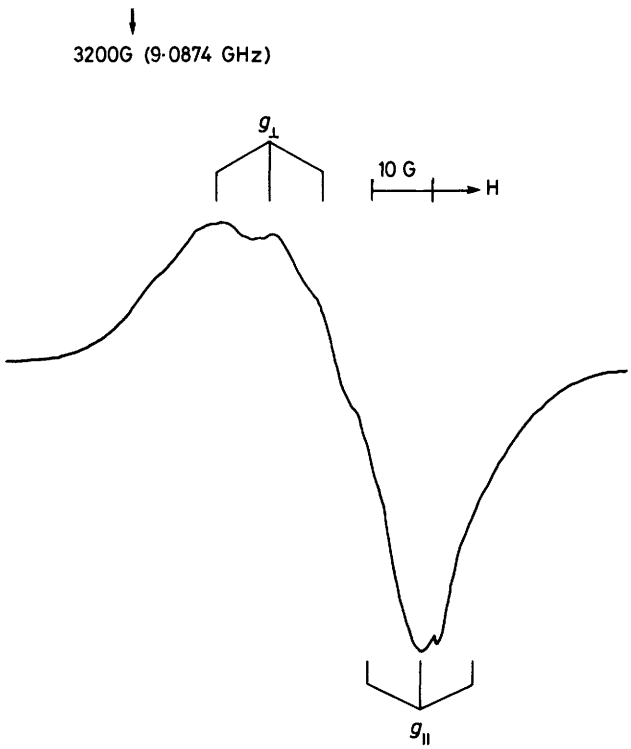
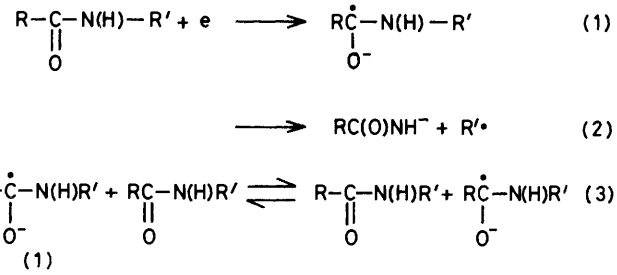
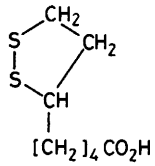


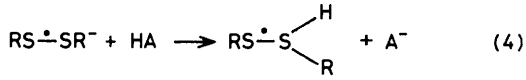
Figure 2. First derivative X-band e.s.r. spectrum for a dilute solution of lysosyme in D_2O + CD_3OD (4 : 1 v/v) after exposure to ^{60}Co γ-rays at 77 K and annealing to remove signals from solvent radicals, showing feature assigned to $(RS^{\therefore}SR)^-$ radical-anions



(1)



(2)



electron capture at the S–S bond. Such terminal groups are, of course, rare in high molecular weight protein units.

One significant observation was that if oxygen was not scrupulously removed, signals characteristic of $RO_2\cdot$ radicals grew in at very low doses prior to the growth of any other radicals. We are investigating the source of these radicals, but we do not consider that they were necessarily formed from protein radicals by the addition of oxygen.

In the absence of oxygen, the rate of growth of e.s.r. signals for equal concentrations of proteins was about the same in the presence or absence of disulphide linkages. This result is, in our view, of considerable significance. The simplest explanation seems to be that once an electron has 'entered' a protein molecule it can move around and selectively seek out the S–S bonds, at which it is preferentially trapped. In the absence of S–S bonds or other electron scavengers, it reacts either with aromatic rings, or probably with amide linkages to give anions, or dissociative electron capture [reactions (1) and (2)].[20] It may also seek out terminal $-NH_3^+$ units. All such radicals would contribute to the free-spin signals observed in the absence of disulphide linkages. This aspect of our work is worthy of more extensive study but, in the meantime, we provisionally conclude that excess electrons are remarkably mobile within proteins prior to being permanently trapped. We suggest that transport occurs *via* electron-transfer of the type (3). Such transfers will be facile provided they occur more rapidly than the time required for radical (1) to relax to its equilibrium conformation (probably pyramidal at the radical centre) or to undergo dissociative electron capture (2). This result may be of importance in the study of electron-transfer reactions of enzymes.[17]

We have previously commented on the apparently high selectivity of electron addition to met-haemoglobin and oxy-haemoglobin under these conditions.[14] Also addition to Mo^{IV} in xanthine oxidase [15] and to the $Cu{-}O_2{-}Cu$ unit in certain haemocyanins [16] occurred with remarkable efficiency. In the present study, we used the Cu^{II} form of superoxide dismutase, which also contains a disulphide linkage, to compare the efficiency of electron capture at the two sites. The results showed a rapid fall in the e.s.r. signal due to Cu^{II} ions prior to the growth of features for $(RSSR)^-$ anions. After complete chemical reduction of the Cu^{II} centres, however, clear features for $(RSSR)^-$ anions could be detected. Similar signals were obtained from the apoenzyme after removal of copper. Once again, electron addition to sites within the protein seems to be remarkably selective and efficient.

Availability of S–S *Units.*—In all cases, electron addition to S–S groups occurred efficiently at 77 K. This result contrasts with results from chemical reduction, in which buried S–S groups are very difficult to reduce, and also with radiolytically induced electron-transfer from radicals such as $Me\dot{C}HOH$ and $\cdot CO_2^-$.[21] In the latter studies, for example, ribonuclease gave no $(RSSR)^-$ anions, whereas bovine serum albumin gave good yields, as judged by optical spectroscopy.[21] This is because the S–S unit in ribonuclease is strongly protected by surrounding protein.

The E.s.r. Data.—That the α^* orbital is well described as primarily 3*p*–3*p* on sulphur has been established by the ^{33}S hyperfine coupling.[11] The e.s.r. spectrum of the radical-anion of cystine formed under comparable conditions is characterised by a hyperfine triplet of *ca.* 8.5 G (Table and Figure 1). Single crystal data are, of course, more precise, but the powder spectrum demonstrates that many of the spectra detected in the present study are close to that expected for $[RCH_2S^{\cdot-}SCH_2R]$ anions. It seems that the preferred conformations for these anions are such that only two of the four β-protons interact strongly with the unpaired electron.

The form of the *g*-tensor components give, in principle, some measure of the degree of twist (θ) of one RS-group relative to the other. For θ 90° symmetry requires that $g_x = g_y$ ($g_\perp$), and the structure requires that $g_\perp > g_\parallel \approx 2.0023$. However, when θ deviates from 90° $g_x \neq g_y$.

In many of our spectra, we have been unable to distinguish between g_x and g_y so θ must be close to 90°. However, in a few instances (Table) rather large deviations from axial symmetry were observed. Presumably, in these cases, the requirements of the protein structure tend to open or close the bond angle. The effect is particularly noticeable for the anion-radicals of α-chymotrypsin, lysozyme, glutathione reductase, and lipoamide dehydrogenase. This hypothesis is nicely verified by results for lipoic acid (2). Here the ring structure restricts θ to 60°, and the e.s.r. spectrum for the anion shows a marked deviation from axial symmetry, with g_x 2.020 and g_y 2.012. It is interesting that, in this case, only one proton coupling of *ca.* 10 G was detected.[5]

Species X.—This species (either $RS^{\cdot-}SR_2$ or $RSS\cdot$ as discussed above) is very well characterised by its e.s.r. spectrum.[5] In all our studies, we searched carefully for this species at 77 K and during annealing studies. For the disulphides, the only positive result was for reduced superoxide dismutase. If our formulation for this species is correct,[5] these results imply a protonation, as in (4). Clearly this will occur most readily for S–S units in hydrophilic regions of the protein. The results suggest that the S–S group in superoxide dismutase is unusual in being relatively readily accessible to solvent molecules or other proton donors.

Acknowledgement

We thank the S.E.R.C. for a grant to D. N. R. R.

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Received 20*th August* 1982; *Paper* 2/1452