Radiation Damage to Proteins: An Electron Paramagnetic Resonance Study

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Exposure of a wide range of proteins to ionizing radiation at low temperatures has led to the following generalizations: (i) Electrons are ejected indiscriminately from any part of the protein. The residual radical cations largely become localized on the backbone amide units, and are trapped thereon by the N–H proton loss. (ii) These centres are characterized by their ^{14}N hyperfine coupling constants and by coupling to the unique C–H proton. This varies in a manner that is characteristic of α-helical regions and β-sheet regions of the protein. (iii) The ejected electrons, in contrast, are very mobile through the protein until they reach good electron traps, such as transition metal ions or disulfide linkages.

Radiation damage to proteins has been the subject of a very wide range of studies, primarily because of its extreme biological importance. The field has been thoroughly reviewed by Garrison [1] and von Sontagg.[2]

Much early work was concerned with aqueous amino acids, but such studies are not very relevant for high molecular weight proteins, because the active groups are quite different. As with most studies on DNA, attention has been largely focused on dilute aqueous solutions. Under these conditions, initial radiation damage is essentially confined to water, and damage to the proteins is indirect, *via* attack by water radicals. As with aqueous DNA, the $^{\bullet}OH$ radical is strongly implicated, and attack is random, except that addition to double bonds is faster than extraction of hydrogen from C–H bonds. Solvated electrons also add to proteins giving a wide range of products. Products also vary greatly depending on the concentration of oxygen.

Since we are concerned with direct radiation damage to proteins, these extensive studies are not directly relevant. We stress, however, that a wide range of products can be isolated and that reactions do not appear to be particularly specific. Key radicals which are commonly formed include the naturally occurring tyrosyl radical (**1**),[3] tryptophanyl radicals,[4] and $RS^{\bullet}$ and $RS^{\bullet}{-}SR^{-}$ radicals.[5,6]

Most studies of direct radiation damage to proteins have been on solids at room temperature, involving chemical analysis. Most enzymes are rapidly deactivated (*G ca.* 1),[7,8] but it is clear that there is generally no highly specific damage to a small number of particular aminoacid residues. The major stable radical detected by EPR spectroscopy at room temperature gives rise to a well defined 20 G doublet. It has been suggested that the type of radical responsible for this doublet is $RCONH\dot{C}R_2$.[9]

There have been few studies of direct radiation damage to proteins at low temperatures. We have used low temperatures to get matrix-isolated 'primary' radical centres that give good EPR spectra. In our initial work on oxyhaemoglobin we showed that electron addition to the $Hb.FeO_2$ unit gave the novel species $Hb.FeO_2^{-}$, but we failed to identify radical species.[10,11] The electron capture seems to be remarkably specific.[12] We have also shown that Fe^{III} centres are converted to Fe^{II} in good yields,[12] that ferryl species add electrons to give Fe^{III}–O units,[13] that RS–SR units readily give $RS^{\bullet}{-}SR^{-}$ electron adducts,[6] and that the Cu–Cu units in haemocyanin add one electron selectively.[14] These studies confirm that once free electrons are located within proteins, they are very mobile and selective in their reactions. Although reaction at the >C=O units of the main chains is expected, this process is not important if there

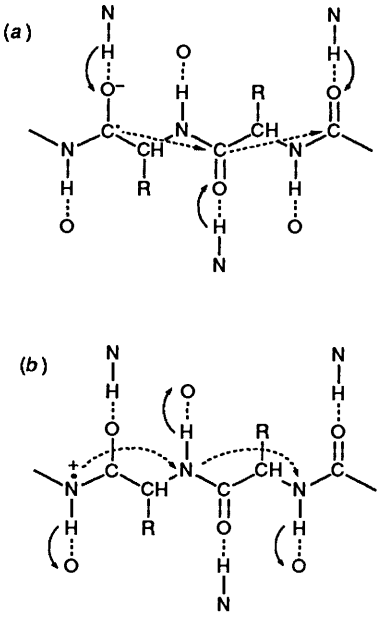

Fig. 1 (*a*) Electron migration (dashed arrows) along a polypeptide chain *vs.* trapping by protonation (curved arrows). (*b*) Hole migration (dashed arrows) *vs.* trapping by deprotonation (curved arrows).

are good electron-capture centres present. We suggest that the electron jumps or tunnels between carbonyl centres, and that localization by protonation at the carbonyl oxygen is a relatively slow process (Fig. 1). This process is comparable with that discussed below for hole centres. It is also closely analogous to the reactions envisaged for electrons in DNA. Here excess electrons are thought to migrate through the base-stacks, favouring C (cytosine) and T (thymine) but only becoming permanently trapped by proton transfer.[15,16]

Thus our evidence suggests extensive migration of electrons through proteins prior to chemical reaction. This accords with many studies involving electron transfer between metal centres in, or on, proteins.[17] If the capture envisaged in Fig. 1 were efficient, these transfers would lead to protein strand-breaks. In marked contrast our preliminary studies of electron-loss centres in proteins suggest that hole migration is usually not extensive. In particular, we have established that Fe^{II} centres in deoxyhaemoglobin do not become Fe^{III} on irradiation at 77 K.[12]

We have invariably detected wing-features (parallel features) that are best assigned to the $M_I = \pm 1$ features for π-N-centred radicals. These, we suggest, are main-chain amido radicals

Table 1 Secondary structural composition of some of the proteins studied in this work

Protein	% α-Helix	% β-Sheet	% Random coil	Ref.
Myoglobin	86	—	14	*a*
Haemoglobin	65	—		*b*
α-Chymotrypsin	21–36	48–60	—	*c*
Lysozyme	52–67	0–33	—	*c*
Concanavalin A	—	45–62	32–42	*c*
Histone II	40	20	40	*d*
Superoxide dismutase	6	50	44	*e*
Protamine	~100	~0	~0	*b*

[a] R. E. Dickerson and I. Geiss, *Haemoglobin*, Benjamin/Cummings, Menlo Park, California, 1983. [b] M. F. Perutz, *Encyclopedia of Polymer Science and Technology*, vol. 11, John Wiley and Sons Inc., New York, London. [c] J. A. Lenstra, *Biochim. Biophys. Acta*, 1977, **491**, 333. [d] P. Y. Chou and G. D. Fasman, *Biochemistry*, 1974, **13**, 211. [e] J. S. Richardson, K. A. Thomas, B. H. Rubin and D. C. Richardson, *Proc. Natl. Acad. Sci. USA*, 1973, **72**, 1349.

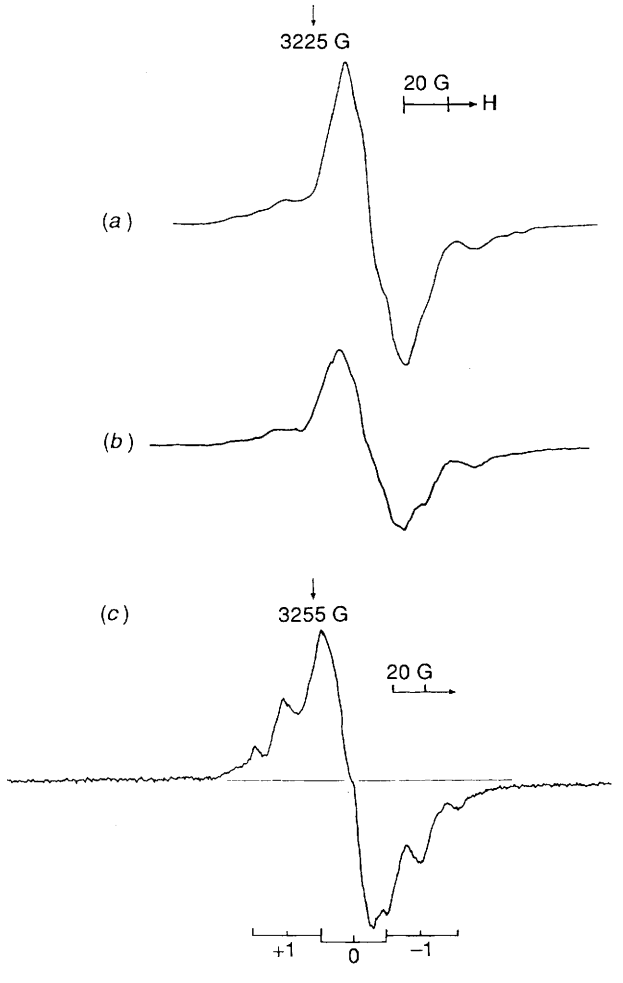

Fig. 2 First derivative X-band EPR spectra for dilute solutions of (*a*) histone II, (*b*) bovine serum albumin and (*c*) α-chymotrypsin, in $K_3Fe(CN)_6$ (1 mmol dm^{-3}) after exposure to ^{60}Co γ-rays at 77 K, and annealing to *ca* 135 K, showing features assigned to the primary electron-loss centres

formed by the proton transfer indicated in Fig. 1(*b*). Thus in this case, we suggest that proton transfer is faster than electron transfer, so that the centres are rapidly localized.

The contrast between reactions (*a*) and (*b*) in Fig. 1 can be understood, in part, in terms of the orbitals involved. For the anion, the orbital is anti-bonding, and relatively expanded. The

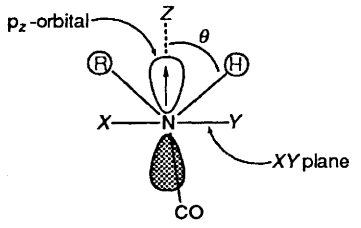

Fig. 3 View along the N–C bond. Amide group is on the *X*–*Y* plane. θ is the dihedral angle between the N p_z-orbital and the C–H bond.

loosely held electron can jump or tunnel rapidly to the equivalent orbital on adjacent amido groups. However, the electron that moves in hole migration is bonding and hence more strongly held to the amido group and is more confined. Since proton transfer within hydrogen-bonded units can be very fast, these differences may well be sufficient to cause the contrast observed.

The aim of this study was to isolate the EPR spectra for the electron-loss centres from those due to electron capture, and hence to attempt to analyse the spectra more fully. In some cases, such as methaemoglobin, this is readily achieved.[10–12] In other cases we have added electron traps such as $Fe(CN)_6^{3-}$ ions. These are less efficient, but do help to suppress central lines from protein radical anions.

Experimental

Human haemoglobin was isolated as previously described.[12] Hemocyanin, myoglobin, α-chymotrypsin, histones, collagen, bovine serum albumin, concanavalin A, and protamine were obtained from Sigma Chemical Company and used without further purification. The metalloproteins were irradiated as their frozen aqueous solutions. Non-metalloproteins were made up in 1 mmol dm^{-3} aqueous solution of $Fe(CN)_6^{3-}$, the Fe^{III} acting as an electron-sink during irradiation.

γ-Radiation and EPR recording were performed as described in ref. 12.

Results and Discussion

The proteins studied, together with some pertinent data are listed in Table 1. Some spectra are given in Figs. 2(*a*)–(*c*). These spectra show weak wing lines assigned to the $M_I = \pm 1(^{14}N)$ features. There are clearly other unidentified features in the central region which cannot be assigned. Simple amido radicals have been previously studied by EPR spectroscopy.[2] These studies show that $A_{iso}(^{14}N) = ca.$ 15 G and $A_{||}(^{14}N) = ca.$ 41 G. Hence we expect $A_{\perp} = ca.$ 2 G which would not be resolved in our spectra. We invariably detect parallel features separated from the centre by *ca.* 40 G in good agreeement with these results.

However there is always one β-proton on the –CHR– unit (two protons for glycine residues) which is expected to give a secondary splitting.

The problem with spectral interpretation is the variability of the β-proton hyperfine coupling with orientation (Fig. 3). This will be a maximum for $\theta = 0°$ varying as $\cos^2\theta$, and falling to near zero for $\theta = 90°$.[18] Thus in the general case, since *A*(average) is probably close to 25 G.[19] $A(^1H)$ may range between 0 and *ca.* 50 G. For an approximately uniform distribution of θ values, there should be initial inflections separated by 50 G marking the onset and loss of absorption, and a feature in the centre corresponding to zero proton coupling.

From liquid phase data however, θ tends to be somewhat more limited in α-helical and β-sheet regions. Judging from Ramachandran maps,[20] α-helical regions tend to favour θ values in the region of 30–70°, whilst β-sheet regions favour θ

values up to 90°. Probably similar limits are observed for random coil domains. For $\theta = 30°$, $A(^1H) = 40$ G. In all EPR spectra, there are weak parallel type features corresponding to a splitting of *ca.* 40 G on the $M_I(^{14}N) = \pm 1$ lines. There is also always a feature close to $A(^1H) = 0$ G although this sometimes appears to be a doublet of up to 10 G splitting. This is favoured by proteins containing β-sheet regions.

Conclusions

We conclude that, for the proteins studied, electron-loss centres are largely confined to the polypeptide backbone amido groups, being trapped by proton transfer from nitrogen. In contrast, electrons are trapped at amido units much less readily, and can migrate to electron-capture centres well removed from the ionization sites.

Acknowledgements

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