TILDEN LECTURE*

Structure and Electron-transfer Reactivity of the Blue Copper Protein Plastocyanin

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1 Introduction

Plastocyanin is a single polypeptide protein of ~99 amino-acids (M. Wt. 10 500) containing a single Cu active site with characteristic type 1 properties, including intense blue colour and small e.p.r. hyperfine coupling ($A_{\parallel}$ close to 0.006 cm^{-1}) for the Cu^{II} state.[1-6] It is involved in electron transport (E^0 370 mV at pH ~7) between photosystems II and I of the chloroplasts in higher plants and algae.[7-10] More specifically its function is to transfer electrons from cytochrome *f* (360 mV) to the chlorophyll-containing pigment $P700^+$ (520 mV) which is a component of photosystem I. Isolation procedures, requiring about one week, are referenced in specific papers.

2 Functions of Plastocyanin

Photosynthesis takes place at highly convoluted thylakoid membranes inside the chloroplast. A thylakoid membrane encloses space so that it has an interior and exterior. Cytochrome *f* is a component part of the membrane-bound cytochrome b_6/f complex, which also includes the Rieske Fe/S protein. It is now known[11,12] that cytochrome *f* is trans-membrane, with the major globular part of the protein (residues 1—250) containing the haem unit, in the interior of the thylakoid (the lumen). Plastocyanin is a mobile molecule also located in the interior. The release of plastocyanin after (mechanical) damage of the membrane, and without use of

* Based on the lecture delivered at a meeting of the Dalton Division of the Royal Society of Chemistry, Scientific Societies' Lecture Theatre, London, on 14th March, 1985.

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[2] A. G. Lappin, in 'Metal Ions in Biological Systems', ed. H. Sigel, M. Dekker, New York, 1981, Vol. 13, pp.15—71.
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[7] W. A. Cramer, W. R. Widger, R. G. Herrmann, and A. Trebst, *TIBS*, 1985, 125.
[8] W. Haehnel, *Annu. Rev. Plant Physiol.*, 1984, **35**, 659.
[9] J. Barber, *Plant Cell Environment*, 1983, **6**, 311.
[10] See also '*Advances in Photosynthetic Research*', Proceedings 6th International Congress on Photosynthesis, Kluwar Academic Publishers Group, Netherlands, 1984 and previous volumes.
[11] D. L. Willey, A. D. Auffret, and J. C. Gray, *Cell*, 1984, **36**, 555.
[12] D. L. Willey, C. J. Howe, A. D. Auffret, C. M. Bowman, T. A. Dyer, and J. C. Gray, *Mol. Gen. Genet.*, 1984, **194**, 416.

detergent, is consistent with it not being membrane-bound. Its function as a diffusible electron carrier between cytochrome *f* and $P700^+$, Figure 1, is comparable to that of cytochrome *c* in the electron transport of bacteria and in the respiratory chain.[8] High plastocyanin concentrations and shielding of surface charges appear to be necessary for the interaction of plastocyanin and $P700^+$ Electron transfer is linked with the generation of protons inside the thylakoid in the water splitting reaction (which involves Mn), and with proton transport for the exterior of the thylakoid by plastoquinol, thereby creating an electrochemical potential gradient for ATP synthesis.

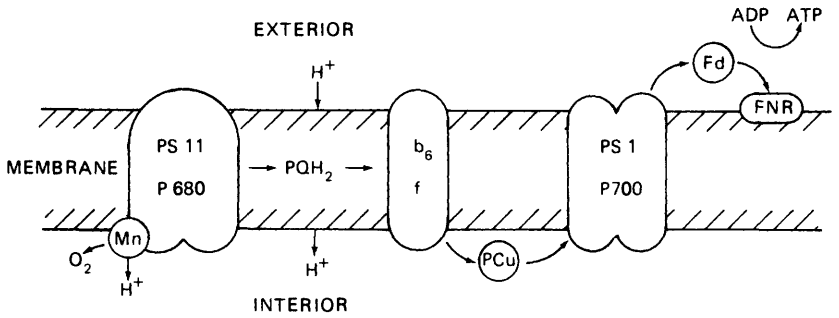

Figure 1 *Schematic representation of photosynthetic electron transport at the thylakoid membrane indicating the function of plastocyanin,* PCu. *Other abbreviations are* Mn *for the* H_2O *splitting complex,* PQH_2 *for plastoquinol,* b_6/f *for the cytochrome complex,* Fd *for ferredoxin,* FNR *for the ferredoxin* $NADP^+$ *reductase, and* ADP *and* ATP *for adenosine di- and tri-phosphates*

3 Aims and Background

Physical properties and the structure of plastocyanin form an important part of what follows. Mechanistic studies described are aimed at better understanding the active-site chemistry, as well as identifying binding sites on the surface of plastocyanin, and in the long term understanding just how electrons are transferred over long distances, with 10—15 Å no longer regarded as exceptional. The *active site* of plastocyanin consists of a single Cu atom and four ligating amino-acid side-chains. Oxidation states I and II, referred to here as PCu^I and PCu^{II} are involved in the redox cycle. A *binding site* is a region on the surface of the protein at which electron transfer with a redox partner occurs. Association of the partner at such a site prior to electron transfer to give a reactant pair (the equivalent of outer-sphere association in the reaction of two metal complexes), does not involve covalent bonding. This contrasts with the case of O_2-carriers and substrate binding (*e.g.* zinc) enzymes, where the active site and binding sites are one and the same, and bond formation occurs. The precise location of electron transfer binding sites is important in order to define the distance over which electrons are transferred. For metalloproteins having an irregular shape with the active site not necessarily at the centre of the molecule, with a non-uniform distribution of groups on the surface,

and non-uniform distribution of charge (which will vary with pH) this is not trivial. Figure 2 which bears some resemblance to plastocyanin illustrates these features. Moreover although a high degree of specificity of binding site is required of natural redox partners, this need not be the case for reactions of proteins with non-natural redox partners, such as small inorganic complexes, when competing sites of varying relevance depending on their distance from the active site may contribute. Most proteins assume a globular shape, with charged residues on the outside. They are soluble in H_2O, and non-aqueous solvents sometimes have disruptive effects. Thus dimethyl sulphoxide (80% v.v. with H_2O) is known to unfold the Fe/S proteins, a property which has been exploited in the extrusion of active site clusters.[13]

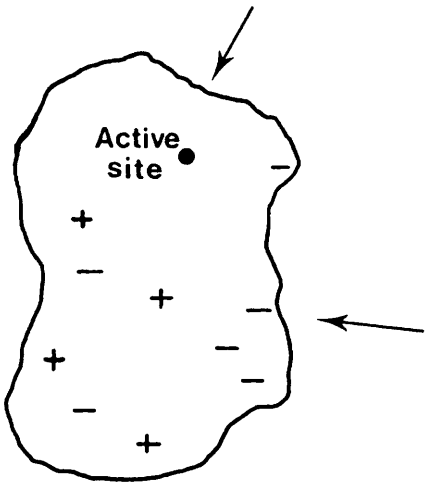

Figure 2 *Representation of a metalloprotein. The two hypothetical binding sites indicated by arrows are different distances from the active site*

The biologically relevant pH for a metalloprotein is not always known with certainty, and for purposes of comparison a pH at or close to 7 is adopted. The pH inside the thylakoid is reported to be about 5.5, although lower values are sometimes indicated. Studies over a range of pH are therefore appropriate, with pH 7 relevant in the overview. At pH 7 aspartic and glutamic acid residues are acid dissociated and have 1− charges, and lysine, arginine, and (unco-ordinated) histidine are protonated and have 1+ charges. In addition, cysteine co-ordinated as thiolate has a 1− charge. Estimates of the total protein charge are approximate only since they take no account of local effects, such as H-bonding between adjacent residues, which can lead to the sharing of H^+, and give abnormal acid dissociation (pK_a) values.

X-Ray crystallographic information from Freeman and colleagues on poplar leaf plastocyanin in both oxidation states have made it structurally one of the best

[13] W. O. Gillum, L. E. Mortenson, J. S. Chen, and R. H. Holm, *J. Am. Chem. Soc.*, 1977, **99**, 584.

understood metalloproteins.[1,14] The question whether the same structure can be assumed to hold in solution is an important one. The n.m.r. method is probably the most powerful technique which can be used to answer this question, and the studies of Williams and colleagues on cytochrome *c* are exemplary in this context. About a half of the 104 amino acids have now been assigned in the ^{1}H n.m.r. spectrum.[15] It would appear that while the structure as a whole remains compact with retention of shape, there are some regions of looser structure which exhibit movement giving differences in solution and solid-state spectra. Studies on plastocyanin have also been carried out, and again the structures in the solid state and in solution appear to be very similar.[16,17] Other techniques which have been used in studies on blue Cu proteins include e.p.r.,[18] EXAFS,[19] and Resonance Raman Spectroscopy.[20,21] Evolutionary aspects have also been considered.[4,22]

Electron-transport metalloproteins have attained a high degree of efficiency; even when the thermodynamic driving force is small electron-transfer reactions are rapid and require rapid mixing or relaxation techniques to monitor their progress. In redox studies stopped-flow spectrophotometry is invariably used to monitor the formation or decay of the blue PCu^{II} absorption at 597 nm (ε 4 500 M^{-1} cm^{-1}). The temperature-jump method continues to be comparatively neglected,[23] in some part because rate laws are more complicated, making it difficult to quantify precisely bimolecular equilibration processes.[24,25]

Because of the size and complexity of metalloproteins it is not generally meaningful at the outset to explore the electron-transfer reactivity between two such molecules without first carrying out exploratory studies. It is now customary to use inorganic complexes as probes for redox reactivity in an initial assessment of each metalloprotein.[2,5,26] Once such an assessment has been carried out it is possible to turn to protein–protein reactions,[27] and ultimately to reactions involving natural protein partners.[28] Even so, the latter can have some problems

[14] J. M. Guss and H. C. Freeman, *J. Mol. Biol.*, 1983, **169**, 521.
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[16] (*a*) D. J. Cookson, M. T. Hayes, and P. E. Wright, *Nature* (London), 1980, 283, 682, *Biochim. Biophys. Acta*, 1980, **591**, 162; (*b*) P. M. Handford, H. A. O. Hill, R. W.-K. Lee, R. A. Henderson, and A. G. Sykes, *J. Inorg. Biochem.*, 1980, **13**, 83.
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[20] T. M. Loehr and J. Sanders-Loehr, *ref.* 17, pp. 115—156.
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[23] S. Wherland and I. Pecht, *Biochemistry*, 1978, **17**, 2585.
[24] M. Goldberg and I. Pecht, *Biochemistry*, 1976, **15**, 4197.
[25] S. K. Chapman, I. Sanemasa, A. D. Watson, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1983, 1949.
[26] S. K. Chapman, I. Sanemasa, A. D. Watson, and A. G. Sykes, *ACS Symp. Ser.* No. 211, ed. M. H. Chisholm, 1983, pp. 177—197.
[27] M. A. Augustin, S. K. Chapman, D. M. Davies, A. D. Watson, and A. G. Sykes, *J. Inorg. Biochem.*, 1984, **20**, 281.
[28] D. Beoku-Betts, S. K. Chapman, C. V. Knox, and A. G. Sykes, *J. Chem. Soc., Chem. Commun.*, 1983, 1150; *Inorg. Chem.*, 1985, **24**, 1677.

(as with cytochrome *f* and plastocyanin), because rate constants are fast and at the upper limit of the stopped-flow range.

4 Amino-acid Sequences

Primary structure information is available for plastocyanin from 14 higher plants, 3 green alga (*Chlorella fusca* and *Scenedesmus obliquus* are referred to in this review) and 1 blue-green alga (*Anabaena variabilis*), all of which have been completely sequenced.[4,14,29–31] In addition a further 42 plastocyanins have been partially sequenced (first 32—40 residues). Sequences which are particularly relevant to this review are shown in Figure 3. Of the 14 completed higher plant sequences 45 residues are invariant (52 with the exclusion of parsley which indicates a remarkable variability for this molecule). Sequence homologies are not as strong if the 3 algae are included when only 24 residues are invariant. Plant plastocyanins normally have 99 residues and algae as many as 105 for *A. variabilis*.[32] The invariant residues include His-37, Cys-84, His-87, and Met-92 which are co-ordinated to the Cu (see below). Variations are likely to be most extensive in the functionally least important regions of the protein. Although residues carrying charge are not always invariant there is with the exception of *A. variabilis* conservation of overall charge, which is estimated to be between -8 and -10 for PCu^{I} at pH 7. The isoelectric point, pI ~ 4.2 for spinach plastocyanin,[33] indicates charge neutrality at pH 4.2. At lower pHs the protein denatures. In sharp contrast *A. variabilis* in the PCu^{I} state has an estimated charge of $+2$ (from the sequence), and from its column behaviour a pI >7. Accordingly *A. variabilis* plastocyanin has quite different properties. The green algal plastocyanins have similar overall charges to those of the higher plants. The deletions at 57 and 58 are an unusual feature, which (surprisingly) have now also been observed for parsley plastocyanin.[29]

The high degree of sequence conservation is significant for a number of reasons. Firstly, it suggests that structural information obtained for poplar plastocyanin should be valid for other members of the family. For a fuller understanding, however, it is now desirable to have crystal structure information for plastocyanin from such diverse sources as parsley and *A. variabilis*. For the present it is assumed that gross overall features of the structure are retained. The close similarity of ^{1}H n.m.r. spectra for PCu^{I} from four different sources are consistent with this.[34] Secondly, it is likely that not just the amino acids at active site, but those constituting the binding site(s) for higher plant and green algal plastocyanins are

[29] The parsley plastocyanin sequence has been determined, R. P. Ambler and A. G. Sykes, unpublished work included in Figure 3.

[30] The *Scenedesmus obliquus* sequence has been determined J. M. Kelly and R. P. Ambler unpublished work included (with permission) in Figure 3.

[31] J. A. M. Ramshaw, in 'Nucleic Acids and Proteins in Plants I, Encyclopedia of Plant Physiology', Vol. 14A, ed. D. Boulter and B. Parthier, Springer Verlag, Berlin, 1982, pp. 229—240.

[32] A. Aitken, *Biochem. J.*, 1975, **149**, 675.

[33] J. A. M. Ramshaw, R. H. Brown, M. D. Scawen, and D. Boulter, *Biochem. Biophys. Acta*, 1973, **303**, 269, see also *ref.* 59.

[34] H. C. Freeman, V. A. Norris, J. A. M. Ramshaw, and P. E. Wright, *FEBS Lett.*, 1978, **86**, 131.

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                                5                   10                  15                  20                  25
A.variabilis  Glu-Thr-Tyr-Thr-Val-Lys-Leu-Gly-Ser-Asp-Lys-Gly-Leu-Leu-Val-Phe-Glu-Pro-Ala-Lys-Leu-Thr-Ile-Lys-Pro-Gly-Asp
S.obliquus          Ala-Asn-Val-Lys-Leu-Gly-Ala-Asp-Ser-Gly-Ala-Leu-Val-Phe-Glu-Pro-Ala-Thr-Val-Thr-Ile-Lys-Ala-Gly-Asp
  Poplar            Ile-Asp-Val-Leu-Leu-Gly-Ala-Asp-Asp-Gly-Ser-Leu-Ala-Phe-Val-Pro-Ser-Glu-Phe-Ser-Ile-Ser-Pro-Gly-Glu
  Spinach           Val-Glu-Val-Leu-Leu-Gly-Gly-Gly-Asp-Gly-Ser-Leu-Ala-Phe-Leu-Pro-Gly-Asp-Phe-Ser-Val-Ala-Ser-Gly-Glu
  Fr. bean          Leu-Glu-Val-Leu-Leu-Gly-Ser-Gly-Asp-Gly-Ser-Leu-Val-Phe-Val-Pro-Ser-Glu-Phe-Ser-Val-Pro-Ser-Gly-Glu
  Parsley           Ala-Glu-Val-Lys-Leu-Gly-Ser-Asp-Asp-Gly-Gly-Leu-Val-Phe-Ser-Pro-Ser-Ser-Phe-Thr-Val-Ala-Ala-Gly-Glu
  [Conservation]                  †  ‡                    ‡     ‡     ‡                                 ‡

                               30                  35                  40                  45                  50
A.variabilis        Thr-Val-Glu-Phe-Leu-Asn-Asn-Lys-Val-Pro-Pro-His-Asn-Val-Val-Phe-Asp-Ala-Ala-Leu-Asn-Pro-Ala-Lys-Ser
S.obliquus          Ser-Val-Thr-Trp-Thr-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Ile-Val-Phe-Asp-Glu-Asp-Ala-Val-Pro-Ala-Gly-Val
  Poplar            Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Ile-Val-Phe-Asp-Glu-Asp-Ser-Ile-Pro-Ser-Gly-Val
  Spinach           Glu-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val-Phe-Asp-Glu-Asp-Glu-Ile-Pro-Ser-Gly-Val
  Fr. bean          Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val-Phe-Asp-Glu-Asp-Glu-Ile-Pro-Ala-Gly-Val
  Parsley           Lys-Ile-Thr-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Ile-Val-Phe-Asp-Glu-Asp-Glu-Val-Pro-Ala-Gly-Val
  [Conservation]       †           †  †  †           †  ‡  ‡  ‡           ‡  ‡  †  †           ‡           †  †

                               55                  60                  65                  70                  75
A.variabilis        Ala-Asp-Leu-Ala-Lys-Ser-Leu-Ser-His-Lys-Gln-Leu-Leu-Met-Ser-Pro-Gly-Gln-Ser-Thr-Ser-Thr-Thr-Phe-Asp
S.obliquus          Asn-Ala-Asp-Ala-Leu-Ser- * - * -His-Asp-Asp-Tyr-Leu-Asn-Ala-Pro-Gly-Glu-Ser-Tyr-Thr-Ala-Lys-Phe-Asp
  Poplar            Asp-Ala-Ser-Lys-Ile-Ser-Met-Ser-Glu-Glu-Asp-Leu-Leu-Asn-Ala-Lys-Gly-Glu-Thr-Phe-Glu-Val-Ala-Leu-Ser
  Spinach           Asp-Ala-Ala-Lys-Ile-Ser-Met-Ser-Glu-Glu-Asp-Leu-Leu-Asn-Ala-Pro-Gly-Glu-Thr-Tyr-Lys-Val-Thr-Leu-Thr
  Fr. bean          Asp-Ala-Val-Lys-Ile-Ser-Met-Pro-Glu-Glu-Glu-Leu-Leu-Asn-Ala-Pro-Gly-Glu-Thr-Tyr-Val-Val-Thr-Leu-Asp
  Parsley           Asn-Ala-Glu-Lys-Ile-Ser- * - *-Gln-Pro-Glu-Tyr-Leu-Asn-Gly-Ala-Gly-Glu-Thr-Tyr-Glu-Val-Thr-Leu-Thr
  [Conservation]              †  †  ‡                          ‡  †           ‡  †                 †     †

                               80                  85                  90                  95
A.variabilis        Ala-Ala-Gly-Glu-Tyr-Thr-Phe-Tyr-Cys-Glu-Pro-His-Arg-Gly-Ala-Gly-Met-Val-Gly-Lys-Ile-Thr-Val-Ala-Gly
S.obliquus          Thr-Ala-Gly-Glu-Tyr-Gly-Tyr-Phe-Cys-Glu-Pro-His-Gln-Gly-Ala-Gly-Met-Val-Gly-Thr-Ile-Val-Gln
  Poplar            Asn-Lys-Gly-Glu-Tyr-Ser-Phe-Tyr-Cys-Ser-Pro-His-Gln-Gly-Ala-Gly-Met-Val-Gly-Lys-Val-Thr-Val-Asn
  Spinach           Glu-Lys-Gly-Thr-Tyr-Lys-Phe-Tyr-Cys-Ser-Pro-His-Gln-Gly-Ala-Gly-Met-Val-Gly-Lys-Val-Thr-Val-Asn
  Fr. bean          Thr-Lys-Gly-Thr-Tyr-Ser-Phe-Tyr-Cys-Ser-Pro-His-Gln-Gly-Ala-Gly-Met-Val-Gly-Lys-Val-Thr-Val-Asn
  Parsley           Glu-Lys-Gly-Thr-Tyr-Lys-Phe-Tyr-Cys-Glu-Pro-His-Ala-Gly-Ala-Gly-Met-Lys-Gly-Glu-Val-Thr-Val-Asn
  [Conservation]          ‡     ‡     †  †  ‡     ‡  ‡     ‡  ‡  ‡  ‡     ‡     †  ‡  ‡
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Figure 3 *Plastocyanin amino-acid sequences referred to in this review. The asterisk* (*) *indicates deletions,* ‡ *positions exhibiting invariance for all* 17 *completed sequences, and* † *additional positions exhibiting invariance for higher plant plastocyanins*

conserved. In this context the conservation of negative charge at positions 42—45 and 59—61, and absence of charge in other regions is noted, Figure 3. Ironically the only higher plant plastocyanin for which the Asp-Glu-Asp-Glu sequence for residues 42—45 is broken is for poplar (45 is Ser),[14] while for parsley negative charge at positions 59—61 is not conserved which follows closely the deletions at 57 and 58.[29] *A. variabilis* is clearly in a category on its own since only 42(Asp) of 42—45 and 59—61 is negatively charged, although residue 85(Glu) compensates towards retention of some negative charge in this locality.[32] This could be significant as will be discussed later. Although no studies on green algal plastocyanins *e.g. S. obliquus* and *C. fusca*, have yet been carried out, these are now important examples in any systematic appraisal of reactivity, because of the versatility in sequence as compared to the higher plant plastocyanins.

5 Crystal Structure

Freeman and colleagues have determined the structure of poplar plastocyanin in the Cu^{II} state to 1.6 Å resolution. Relevant information is summarized only briefly here, and the papers of Freeman [1,14] are strongly recommended for further reading. The Cu is co-ordinated to N-atoms of His-37 and His-87 and the S-atoms of Cys-84 and Met-92 in an irregular tetrahedral co-ordination geometry (Figure 4). Two of the bond angles at the Cu differ by more than 20° from a regular tetrahedron. The two imidazole Cu–N bonds at 2.10 and 2.04 Å, and the thiolate Cu–S (Cys) bond at 2.13 Å may be regarded as normal, but the thio-ether Cu–S(Met) distance (2.90 Å)

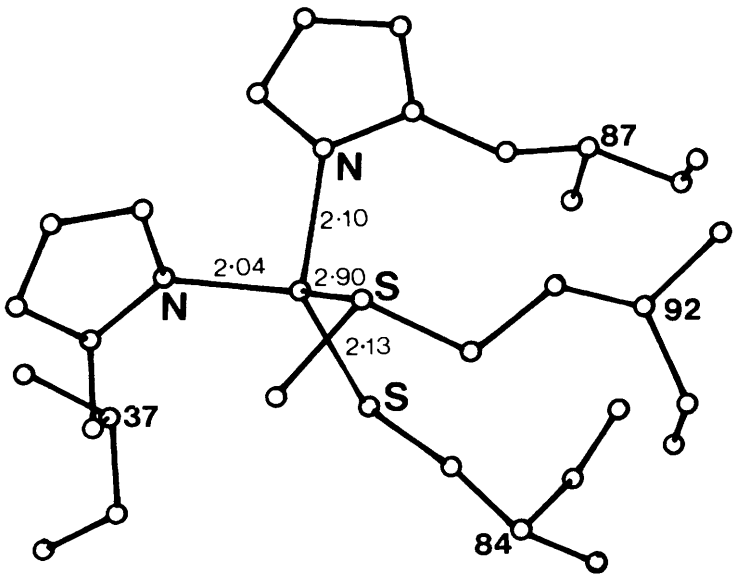

Figure 4 *The active site of plastocyanin*, PCu^{II}

is sufficiently long as to raise questions as to its relevance. It is not detected by EXAFS measurements.[35] There are no important changes in the PCu^{II} crystal structure at pH 4.2 as compared to 6.0, and the geometry of the active site does not change.[1] It is estimated that 36% by volume of the crystal is occupied by solvent H_2O. Forty-four water molecules have been identified, but none of these is found at the active site or elsewhere in the interior of the protein.[14]

The molecule has the shape of a slightly flattened cylinder of approximate dimensions 40 × 32 × 28 Å, the 40 Å distance corresponding to what is sometimes referred to as a north–south axis. The Cu atom is buried 6 Å in the interior at the north end. There are eight strands of polypeptide chain which are connected by seven loops at the ends of the cylinder. Seven of the strands have substantial β-character, and strand five is irregular and contains the only helical structure (about 1.5 turns). As already mentioned *S. obliquus*, *C. fusca*, and parsley plastocyanins have deletions at 57 and 58. The two green algae retain a 2− charge in this locality but parsley has only one negative charge at Glu-61 or Glu-59 if the two deletions are not included in the numbering. A *normal* view of the molecule which is often used is shown in Figure 5. The right-hand side, which includes the 42—45 and 59—61 negative patches already referred to, is sometimes referred to as the east side. Some care is required because rotation about the north–south axis could lead to ambiguity in description.

All the charged residues and the majority of uncharged polar residues (Ser, Thr, Asn, and Gln) are exposed to solvent. The majority, but not all, of the non-polar and aromatic residues are buried within the molecule. It has been noted that the exposed edge of the His-87 imidazole ring is level with the molecular surface at the northern extremity. This partly exposed His-87 is surrounded by conserved non-polar groups, and constitutes the so-called hydrophobic patch. The Tyr-83 residue is exposed to solvent, Tyr-70 has one side exposed, whereas Tyr-80 is on the inside of the protein and is H-bonded to a peptide carbonyl group.

There is a striking imbalance in charge on the surface of plastocyanin. None of the ten charged residues which are conserved in plant plastocyanins (eight Glu/Asp and two Lys) occur in the northern quarter of the molecule. It is important to note that the highly conserved 42—45 and 59—61 regions are concentrated in two kinks in the protein backbone. These occur either side of Tyr-83 and at pH ~ 7 their negatively charged carboxylates are directed into the solvent and form an elongated region of negative charge.

A structure analysis of poplar PCu^{I} has been carried out at six pHs in the range 3.9 to 7.9. At pH 7 the co-ordination geometry is very like that of PCu^{II}. Active-site structural changes observed at lower pHs will be referred to later.

6 Spectroscopic Properties

Studies on Co^{II}-substituted plastocyanin in which the Cu is replaced by Co^{II} lead to the assignment of the dominant 597 nm band (Figure 6) as S(Cys-84) → Cu^{II} charge-

[35] R. A. Scott, J. E. Hahn, S. Doniach, H. C. Freeman, and K. O. Hodgson, *J. Am. Chem. Soc.*, 1982, **104**, 5364.

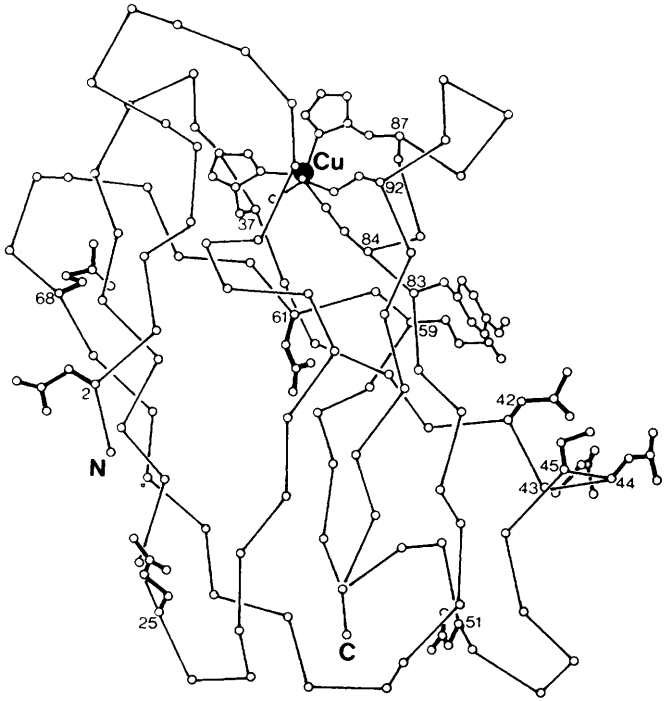

Figure 5 *The structure of poplar plastocyanin,* PCu^{II}, *as obtained by Freeman*[1,14]

transfer.[36,37] As many as six other satellite bands have been reported at 464, 551, 739, 838, 971, and 1 818 nm.[38] Assignments made involve the cysteine and histidine ligands. While the S (Met-92) is believed to make a weak but definite contribution to the ligand field at the Cu atom, the analysis reported by Penfield *et al.*[39] does not reveal any S(Met-92) to Cu^{II} charge-transfer component in the electronic spectrum. Additional detail (often ignored) is observed in the near-u.v. spectrum of PCu^{I} and PCu^{II}, with peaks or shoulders at 284, 278, 273, 269, 266, 259, 252, and 248 nm (Figure 6). The peaks at 284 and 278 nm have been assigned to tyrosines, and the rest to phenylalanine residues.[40] Absorbances for PCu^{I} are (for spinach) ~70% those observed for the PCu^{II} protein; PCu^{I} does not absorb in the visible.[41]

The ^{1}H and ^{13}C n.m.r. method can be used to study amino acids close to the Cu

[36] D. R. McMillin, R. C. Rosenberg, and H. B. Gray, *Proc. Natl. Acad. Sci. USA*, 1974, **71**, 1339 and 4760.
[37] E. I. Solomon, J. Rawlings, D. R. McMillin, P. J. Stevens, and H. B. Gray, *J. Am. Chem. Soc.*, 1976, **98**, 8046.
[38] E. I. Solomon, J. W. Hare, and H. B. Gray, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 1389.
[39] K. W. Penfield, R. R. Gray, R. S. Himmelwright, N. C. Eickman, V. A. Norris, H. C. Freeman, and E. I. Solomon, *J. Am. Chem. Soc.*, 1981, **103**, 4382.
[40] J. W. Donovan in 'Physical Principles and Techniques in Protein Chemistry', ed. S. J. Leach, Academic Press, New York, 1979, Part A, pp. 102—172.
[41] E. L. Gross, G. P. Anderson, S. L. Ketchner, and J. E. Draheim, *Biochim. Biophys. Acta*, 1985, in press, and personal communication from Professor E. L. Gross.

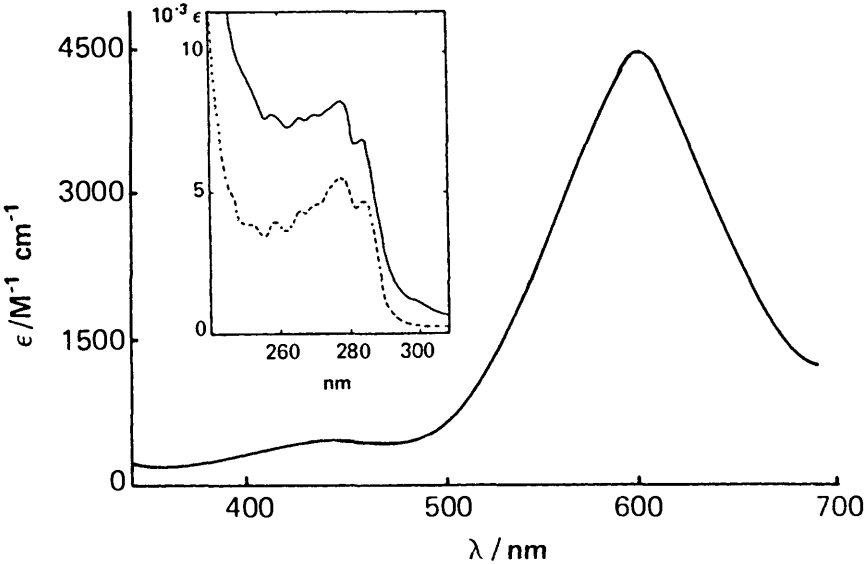

Figure 6 *Visible absorbance spectrum for plastocyanin,* PCu^{II}. *For the u.v. range (inset) spectra are for* PCu^{I} (–) *and* PCu^{II} (– – –). *The u.v. spectra*[41] *are specific to spinach plastocyanin, since aromatic composition is important*

active site in the reduced but not (due to line broadening) oxidized form of protein. Resonances which are most readily identified are those due to the methyl of the methionine, and the histidine, tyrosine, and phenylanlanine aromatic-ring protons.[42] A number of N–H peptide signals remain unexchanged in D_2O consistent with a compact inaccessible core to the protein. It has also been demonstrated that the Cu^{II} at the active site of the type-1 proteins is inaccessible to solvent H_2O.[43]

Adequate modelling of the tetrahedral Cu^{II} site in low M.Wt. complexes is difficult, and although the intense blue colour has been reported for a tetrahedral pyrazolylborate complex having N_2S_2 and N_3S co-ordination,[44] and e.p.r. spectra have *g*-values that match those of the blue Cu proteins, their Cu hyperfine $A_{\|}$ values are normal rather than very small as for the proteins. The difficulties in precise modelling are the distorted tetrahedral angles and long Cu to thioether distance.

7 Reduction Potentials

Values of E^0 in the range 347 to 395 mV have been reported for PCu^{II}/PCu^{I} at pH 7.0, which is high compared to the aqua Cu^{II}/Cu^{I} couple (115 mV) and corresponds to a stabilization of the Cu^{I} state. Until recently such values were determined using

[42] E. L. Ulrich and J. L. Markley, *Coord. Chem. Rev.*, 1978, **27**, 109.
[43] N. Boden, M. C. Holmes, and P. F. Knowles, *Biochem. Biophys. Res. Commun.*, 1974, **57**, 847; L. Avigliano, A. Finazzi-Agro, and B. Mondovi, *FEBS Lett.*, 1974, **38**, 205.
[44] J. S. Thompson, T. J. Marks, and J. A. Ibers, *J. Am. Chem. Soc.*, 1979, **101**, 4180.

a mediator, generally the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ couple,[45] because of the slow response of proteins at electrode surfaces. Direct non-mediated electrochemistry has now been observed at an edge-orientated pyrolytic graphite electrode, which when subjected to a standard polishing procedure in air gives C–O functional groups capable of interacting with the protein (25 μM) in the presence of Mg^{2+} (<5mM).[46] By attaching positively charged Cr^{III} complexes to the graphite electrode surface the electrode becomes fully reversible to the plastocyanin couple, but not to cytochrome *c* which is positively charged.[46] In 5 mM buffer/1 mM KCl solution at 3 °C the reduction potential for spinach is 375 mV. Katoh *et al.*[47] first reported an increase in E^0 with decreasing pH for spinach plastocyanin, which has been confirmed in kinetic studies with parsley,[48] Figure 7, and potentiometric studies on marrow plastocyanin.[49] At pH 4.2 the higher E^0 of 430 mV is the result of changes at the active site of the Cu^I protein.

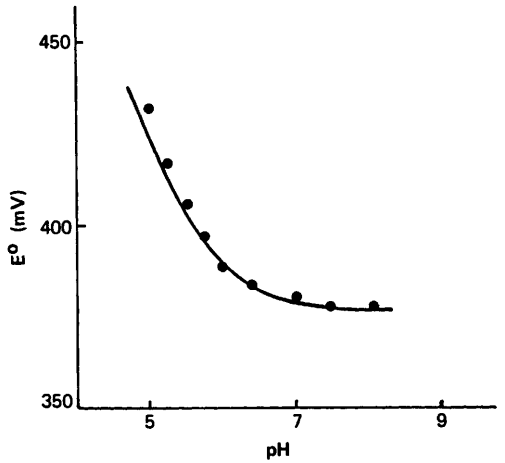

Figure 7 *Variation of E^0 with pH for parsley plastocyanin*

8 Other Single (Type 1) Blue Cu Proteins

All single type-1 proteins in the Cu^{II} state have in common the intense blue colour and exceptionally small hyperfine-splitting in the e.p.r. spectrum. Table 1 summarizes properties of some of these proteins.[2,6] Other proteins of the type which have been characterized include plantacyanin,[50] mung bean blue,[51]

[45] G. W. Pettigrew, D. A. Leitch, and G. R. Moore, *Biochim. Biophys. Acta.* 1983, **725**, 409.
[46] F. A. Armstrong, H. A. O. Hill, B. N. Oliver, and D. Whitford, *J. Am. Chem. Soc.*, 1985, **107**, 1473 and F. A. Armstrong, P. A. Cox, H. A. O. Hill, B. N. Oliver, and A. A. Williams, *J. Chem. Soc., Chem. Commun.*, in press.
[47] S. Katoh, I. Shiratori, and A. Takamiya, *J. Biochem. (Tokyo)*, 1962, **51**, 32.
[48] M. G. Segal and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **100**, 4585.
[49] M. D. Scawen, E. J. Hewitt, and D. M. James, *Phytochemistry*, 1975, **14**, 1225, and *ref.* 4, p. 12.
[50] K. A. Markossian, V. T. Aikcazyan, and R. M. Nalbandyan, *Biochim. Biophys. Acta*, 1974, **359**, 47; V. T. Aikcazyan and R. M. Nalbandyan, *FEBS Lett.*, 1975, **55**, 272; and T. Sakurai, H. Okamoto, K. Kawahara, and A. Nakahara, *FEBS Lett.*, 1982, **147**, 220.
[51] H. Schichi and D. P. Hackett, *Arch. Biochem. Biophys.*, 1963, **100**, 183.

amicyanin,[52,53] mavicyanin,[54] and basic blue from cucumber.[55] In addition, multi-copper oxidases such as the laccases, ceruloplasmin, and ascorbic acid oxidase have a type-1 Cu as well as type-2 and type-3 active sites.[6,56] As can be seen from Table 1 the type-1 proteins have a variety of different origins, and while their function almost certainly remains associated with electron transport, only plastocyanin is involved in photosynthesis. Many of the properties differ considerably from those of plastocyanin. The sequence of azurin from ten different bacterial sources has been determined and is also highly conserved.[2] Crystal structure information on azurin from *Pseudomonas aeruginosa* (2.7 Å resolution)[57] and of azurin from *Alcaligenes denitrificans* to 2.5 Å has been obtained.[58] The structure conforms to the plastocyanin model. Structure homologies between the plastocyanins and azurins have been noted.[59] The co-ordination spheres and position of the Cu ($\sim$7 Å buried) are also similar to plastocyanin. The most striking departure from the plastocyanin structure is the addition of a flap between residues 53 and 78. This flap, which includes some helical turns and seems to be an extension of the irregular fifth strand, hangs outside the body of the rest of the molecule in the region of the east site. Active site co-ordination of the Cu is to His-46, Cys-112, His-117, and Met-121. The absorption maximum for azurin at 625 nm is appreciably shifted compared to that of plastocyanin at 597 nm. Additional weak co-ordination of an adjacent carboxyl of the peptide chain, which approaches to within $\sim$3.2 Å of the Cu, is possible. Azurin, like plastocyanin, has a northern hydrophobic surface. There is no negatively charged region and the charge is -2 and -1 for the two states, assuming only one of the two unco-ordinated histidines contributes a $1+$ charge.

Differences are to be noted in the case of stellacyanin which has no methionine,[60] and umecyanin which although it has three methionines has none in the sequence after residue-74.[61] In both cases therefore the fourth ligand is unlikely to be methionine.[62] It appears that in the case of stellacyanin the fourth ligand may be a disulphide, which it has been suggested[63] is formed during the isolation procedure, and that two thiolate ligands are present in the original protein. This remains to be confirmed. Rusticyanin is remarkable in that *in vivo* it is reduced by Fe^{2+},[64] and it

[52] Y. Morita, A. Wadano, and S. Ida, *Agr. Biol. Chem.*, 1981, **101**, 502; T. van Houwelingen, G. W. Canters, J. A. Duine, J. Frank, and G. Stobbelaar, *Rev. Port. Quim.*, 1985, **27**, 177.
[53] J. Tobaria and Y. Harada, *Biochem. Biophys. Res. Commun.*, 1981, **101**, 502; and T. van Houwelingen, G. W. Canters, J. A. Duine, J. Frank, and G. Stobbelaar, *Rev. Port. Quim.*, 1985, **27**, 177.
[54] A. Marchesini, M. Minelli, H. Merkle, and P. M. Koneck, *Eur. J. Biochem.*, 1979, **101**, 77.
[55] M. Muraka, G. S. Begg, F. Lambrou, B. Leslie, R. J. Simpson, H. C. Freeman, and J. F. Morgan, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 6434.
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[57] E. T. Adman and L. H. Jensen, *Jsr. J. Chem.*, 1981, **21**, 8.
[58] G. E. Norris, B. F. Anderson, and E. N. Baker, *J. Mol. Biol.*, 1983, **165**, 501.
[59] D. Boulter, B. G. Haslett, D. Peacock, J. A. M. Ramshaw, and M. D. Scawen, *Int. Rev. Biochem.*, 1977, **13**, 1.
[60] C. Bergman, E.-K. Gandvik, P. O. Nyman, and L. Stid, *Biochem. Biophys. Res. Commun.*, 1977, **77**, 1052.
[61] C. Bergman, Ph.D. Thesis, Chalmers University of Technology, Goteborg, 1980.
[62] C. Bergan and P. O. Nyman, unpublished work.
[63] H. A. O. Hill and W.-K. Lee, *Biochem. Soc. Trans.*, 1979, **7**, 733.
[64] J. G. Cobley and B. A. Haddock, *FEBS Lett.*, 1975, **60**, 29.

Table 1 *A comparison of properties of single blue Cu proteins*

Protein	*Source*	*M.Wt.*	*No. of amino-acids*	*pI*	E^0/mV	$\lambda_{max.}(\varepsilon)$/nm(/$M^{-1}$ cm^{-1})
Plastocyanin	Chloroplasts, plants/algae	10 500	99	4.2[a]	370[b]	597 (4 500)
Azurin	*Pseudomonas aeruginosa*[c]	14 000	128	5.4	330[d]	625 (4 800)
Stellacyanin	Lacquer tree[e]	20 000[f]	107	9.86	184	608 (4 080)
Rusticyanin	*Thiobac. ferro-oxidans*	16 000	159	9.1	680[g]	597 (1 950)
Umecyanin	Horse-radish roots	14 600	125	5.85	283	610 (3 400)

[a] Spinach. [b] At pH 7. [c] Other bacteria *also.* [d] At pH 7. 350 mV at pH 5. [e] *Rhus vernicifera.* [f] 40% carbohydrate. [g] pH 2.

normally functions at pH 2, at which pH no negatively charged residues can be present on the surface. Plastocyanin is denatured at pH < 4. The high E^0 for rusticyanin (680 mV)[65] has not so far been explained. The close proximity of Cys-127, His-132, and Met-137 in the sequence implicates these residues in binding the Cu.[66] There are four other histidines, two methionines, and a number of aspartates in the sequence, one or more of which could also be co-ordinated to the Cu.[66]

Kinetic studies involving umecyanin and rusticyanin are at present underway.

9 EXAFS Studies

Solution and crystal studies for plastocyanin have yielded values in satisfactory agreement with Freeman's *X*-ray crystallographic Cu–N(His) and Cu–S(Cys) distances, Table 2.[67] The long Cu–S(Met) distance is not detected by EXAFS measurements.[35] Information obtained for azurin,[68,69] stellacyanin,[68] and umecyanin,[69] is also indicated in Table 2. Co-ordination of the Cu^{II} to two histidines (average Cu–N distance 1.99 Å) and one thiolate Cu–S (average distance 2.12 Å) is indicated from the fitting. Upon reduction the Cu–N and Cu–S distances increase by ~0.05 Å and ~0.10 Å respectively. A reasonable description of the redox process might therefore be that there is little angular rearrangement of ligands and only small changes in the metal–ligand bond distances. The change in u.v.–visible spectrum observed for UCu^{II} on adjusting the pH from 7.0 (peak at 610 nm) to 10.7 (peak at 580 nm) is not reflected in any differences in EXAFS bond distances, which remain (within experimental error) 1.99 and 2.13 Å respectively. This suggests some minor change, possibly the approach of a fifth ligand (an amine?), which does not establish itself as a fully co-ordinated group. Alternatively a pH-controlled conformational change effective at the Cu^{II} active site could be the explanation.

Table 2 *EXAFS solution studies. In each case a satisfactory fit is obtained for two histidines and one cysteine co-ordinated to the* Cu. *Bond lengths for* Cu–N *(average) and* Cu–S *are given*

	Oxidized		*Reduced*	
	Cu–N(His)/Å	Cu–S(Cys)/Å	Cu–N(His)/Å	Cu–S(Cys)/Å
PCu^{II}	1.97	2.11	2.05	2.22
ACu^{II}	1.97	2.2	2.00	2.22
SCu^{II}	2.04	2.11	2.07	2.25
UCu^{II} [a]	1.99	2.13	2.03	2.21

[a] pH 7.5. Identical results (±0.03 Å) at pH 10.5

[65] W. J. Ingledew, *Biochim. Biophys. Acta*, 1980, **590**, 141.
[66] R. P. Ambler and W. J. Ingledew, unpublished results, personal communication Dr. W. J. Ingledew.
[67] T. D. Tullius and K. O. Hodgson referred to in *ref.* 19.
[68] T. D. Tullius, P. Frank, and K. O. Hodgson, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 4069.
[69] S. K. Chapman, J. McGinnis, J. D. Sinclair-Day, A. G. Sykes, P.-I. Ohlsson, K.-G. Paul, and W. H. Orme-Johnson, to be published.

10 Apoplastocyanin

Apoplastocyanin has been prepared by 7h dialysis of 0.1 mM spinach PCu^{I} against 50 mM KCN and 50 mM phosphate buffer at pH 7.1 under argon.[70] Thiourea at pH 4—5 has been used successfully to remove Cu^{I} from azurin.[71] Crystals of poplar PCu^{II} have been soaked in a sequence of solutions to effect the Cu^{II} to Cu^{I} reduction (ascorbate), and then cyanide (0.15 M) for much longer time intervals (5, 17, and 72 h) to remove the Cu^{I} at pH 5.3 in 5 M phosphate buffer.[72] The crystal structure of the poplar apoplastocyanin to 1.8 Å resolution resembles that of the holoprotein, thus suggesting that the geometry of the type-1 site is imposed by the polypeptide.[72] A rotation of the His-87 ring by 180° appears to facilitate access to the Cu site, and re-entry of the Cu by a reverse process in a trap-door type mechanism has been proposed.

Quantitative kinetic study of the reformation of the Cu protein is not easy as has been illustrated by studies of McMillin and colleagues on the reconstitution of azurin Cu^{II}.[71] One of the problems is the form in which the free Cu is made available at the pHs under investigation. In the azurin studies imidazole and 1-methylimidazole buffers (0.1 M) in the pH range 7—9 were used, with 1 mM Cu^{II} and apoprotein at 2×10^{-5}M. Under these conditions it has been estimated that ~90% of the Cu^{II} exists in solution as the tetrakisimidazole complex. Uptake of Cu occurs in a stepwise fashion, and at least two intermediates have been proposed. In the reconstitution of spinach apoplastocyanin, thionein has been used to complex free Cu^{II}, and $Cu^{I}(thiourea)_3^{+}$ was found to be a convenient source of Cu^{I}.[70] No kinetic studies were reported.

11 Inorganic Complexes as Redox Partners

It is preferable to use substitution inert complexes. Even when there is only a small thermodynamic driving force reactions are rapid and well into the stopped-flow range. The Co^{III}/Co^{II} couple is sometimes an exception because of its unfavourable self-exchange characteristics. Reduction potentials for complexes employed as redox partners for type-1 proteins, in most cases at pH 7, are listed in Table 3.[73] Both states of $[Ru(NH_3)_5py]^{3+/2+}$ and $[Fe(CN)_6]^{3-/4-}$ have been used, where the reaction is driven to completion in the desired direction by having the inorganic complex in sufficiently large excess. Complexes such as $[Fe(edta)]^{2-}$, $[Co(terpy)_2]^{2+}$, and $[Co(phen)_3]^{2+}$, although labile, can be retained in solution by having an excess (say 3:1) of chelating ligand present.

Incorrect interpretation of kinetics can result if a reaction does not proceed to >90% completion or the full rate laws are not used. A particularly striking example of this is the $[Fe(CN)_6]^{4-}$ reduction of cytochrome *c*(III).[74]

[70] S. Brutsch, H.-J. Hartmann, and U. Weser, *Inorg. Chim. Acta*, 1984, **92**, 147.
[71] J. A. Blaszak, D. R. McMillin, A. T. Thornton, and D. L. Tennent, *J. Biol. Chem.*, 1984, **259**, 2822.
[72] T. P. J. Garrett, D. J. Clingeleffer, J. M. Guss, S. J. Rogers, and H. C. Freeman, *J. Biol. Chem.*, 1984, **259**, 2822.
[73] S. K. Chapman, I. Sanemasa, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1983, 2549.
[74] J. Butler, D. M. Davies, and A. G. Sykes, *J. Inorg. Biochem.*, 1981, **15**, 41.

Table 3 *Reduction potentials of inorganic couples (E^0) relevant to studies with type-1* Cu *proteins*

	E^0/mV
$[Fe(edta)]^{1-/2-a}$	120
$[Co(terpy)_2]^{3+/2+}$	270
$[Ru(NH_3)_5py]^{3+/2+}$	273
$[Co(phen)_3]^{3+/2+}$	370
$[Fe(CN)_6]^{3-/4-}$	410
$[(NC)_5FeCNCo(CN)_5]^{5-/6-b}$	460
$[Ru(NH_3)_5py]^{3+/2+}$	273
$[Co(C_2O_4)_3]^{3-/4-}$	570
$[Co(dipic)_2]^{1-/2-c}$	747

[a] edta probably quinquedentate with H_2O in sixth position. [b] *Ref.* 80. [c] Re-determined by N. H. Williams and J. K. Yandell, *Aust. J. Chem.*, 1983, **36**, 2377. Earlier value 400 mV
edta = ethylenediaminetetra-acetate; terpy = terpyridine; py = pyridine; phen = 1,10-phenanthroline; dipic = pyridine-2,6-dicarboxylate

With the complex $[Co(4,7\text{-DPSphen})_3]^{3-}$, where 4,7-DPSphen is 4,7-disphenylsulphonate-1,10-phenanthroline, difficulties have been experienced in obtaining reproducible data, and in interpreting results in a self-consistent manner.[75] This complex is therefore excluded from the present discussion.

12 Kinetic Studies

A range of buffers ($\sim 10^{-2}$M) have been used for pHs in the range 4—10 relevant to metalloprotein reactions.[76] Normally there is satisfactory reproducibility in buffer overlap regions. Ionic strengths are generally adjusted to 0.10 M with NaCl, and the temperature is 25 °C unless otherwise stated. Protein concentrations of 10^{-5}M are widely used, and can be as low as 10^{-7}M for the cytochromes which have intense redox-state-dependent Soret bands. Use of the stopped-flow method as opposed to conventional spectrophotometry also helps to conserve protein. Inorganic complexes, *e.g.*, $[Fe(CN)_6]^{3-}$ in equation (1), are used in >10-fold excess of the protein.

$$PCu^{I} + [Fe(CN)_6]^{3-} \longrightarrow PCu^{II} + [Fe(CN)_6]^{4-} \quad (1)$$

First-order plots give k_{obs}, from which the dependence $k_{obs} = k[Fe(CN)_6^{3-}]$ can be demonstrated. This establishes the simple rate law (2),

$$\text{Rate} = k[PCu^{I}][Fe(CN)_6^{3-}] \quad (2)$$

where k is the second-order rate constant.

[75] (*a*) A. G. Lappin, M. G. Segal, D. C. Weatherburn, and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **101**, 2297, and (*b*) recent studies J. D. Sinclair-Day and A. G. Sykes, unpublished work.
[76] See *e.g. ref.* 28 and preceding papers in this series.

Rate constants for the $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidations of PCu^I from parsley, spinach, and French bean are in good agreement with a spread of less than a factor of two in each case, Table 4.[48,77,78] Conservation of charge at 8−, 9−, and 9− respectively for the PCu^I proteins is noted. Rate constants for PCu^I from *A. variabilis*, charge 2+, indicate a different pattern consistent with the different overall charge.[79]

Table 4 *A comparison of rate constants* (25 °C) *for the* $[Fe(CN)_6]^{3-}$ (k_{Fe}) *and* $[Co(phen)_3]^{3+}$ (k_{Co}) *oxidation of plastocyanin* PCu^I *from different sources at* pH 7.5, I = 0.10 M (NaCl)

Source of PCu^I	$k_{Fe}/M^{-1}\ s^{-1}$	$k_{Co}/M^{-1}\ s^{-1}$	k_{Fe}/k_{Co}
Parsley	94 000	3 000	31
Spinach	85 000	2 500	34
French Bean	58 000	4 700	12
Anabaena variabilis	650 000	680	950

With $[Co(phen)_3]^{3+}$ as oxidant, rate constants k_{obs} give a less than first-order dependence on $[Co(phen)_3^{3+}]$ on increasing the latter to $\sim 3 \times 10^{-3}$M.[48] Instead equation (3) holds

$$k_{obs} = \frac{Kk_{et}[Co^{III}]}{1 + K[Co^{III}]} \tag{3}$$

and the reaction is said to exhibit limiting kinetics where in the present case the limit would only be attained at much higher $[Co(phen)_3^{3+}]$. Equilibrium and rate constants K and k_{et} defined in (4) and (5),

$$PCu^I + Co^{III} \overset{K}{\rightleftharpoons} PCu^I, Co^{III} \tag{4}$$

$$PCu^I, Co^{III} \xrightarrow{k_{et}} PCu^{II} + Co^{II} \tag{5}$$

can be obtained from a graph of $(k_{obs})^{-1}$ against $[Co(phen)_3^{3+}]^{-1}$. Although a satisfactory fit is obtained, the rate law may not be as simple as indicated, and in keeping with later discussion, a linear dependence of k_{obs} on $[Co^{III}]$ may remain at high $[Co^{III}]$. Data for parsley,[48] spinach,[48,47] and French bean[78] PCu^I give a satisfactory fit to (3), with K/M^{-1} at 25 °C 167 and 389 for parsley and spinach respectively, but with *A. variabilis* PCu^I a plot of k_{obs} against $[Co(phen)_3^{3+}]$ is linear, and $K[Co(phen)_3^{3+}]$ is not influential in the denominator of (3), *i.e.* K is small. In such instances second-order rate constants (k) can be equated to Kk_{et} in a two-step process (4) and (5), which is perfectly reasonable as long as rate constants for the association process are very much greater than k_{et}. This interpretation is supported by studies with $[Fe(CN)_6]^{3-}$ and

[77] J. D. Sinclair-Day and A. G. Sykes, to be published.
[78] G. C. King and P. E. Wright, unpublished work.
[79] J. A. Chambers, M. P. Jackman, J. D. Sinclair-Day, M. J. Sisley, and A. G. Sykes, to be published.

$[(NC)_5FeCNCo(CN)_5]^{5-}$ as oxidants for parsley PCu^{I} when limiting kinetics are not observed. However the $\Delta H^{\ddagger}$s for k are negative (-2.9 and -3.3 kcal mol^{-1} respectively) consistent with two-stage processes involving association (K), which presumably has a negative ΔH^{0}, prior to electron transfer (k_{et}).[80] In fact with $[Co(phen)_3]^{3+}$ as oxidant an additional step has to be included alongside (4) and (5), with modification of (3), as will emerge later.

The importance of electrostatics, and its influence on reactions of the type (4) is illustrated in these studies. In addition to the studies with $[Fe(CN)_6]^{3-}$ and $[(NC)_5FeCNCo(CN)_5]^{5-}$ limiting kinetics are not observed with $[Co(dipic)_2]^{-}$, or $[Co(bipy)_2(O_2CMe)_2]^{+}$ as oxidants or with $[Fe(CN)_6]^{4-}$ and $[Ru(NH_3)_5py]^{2+}$ as reductants for PCu^{II}.[81]

Excluding the case of $[Co(4,7\text{-DPSphen})_3]^{3-}$ the $[Co(phen)_3]^{3+}$ study provides the only example so far of limiting kinetics with plastocyanin. Values of K obtained are small and the fact that the solution composition changes on replacing a 1:1 by a 3:1 electrolyte raises some questions. However K increases significantly on decreasing the ionic strength, making it more easily detectable.[78] The converse also applies and K is not detectable at $I = 0.5$ M.[82] Also relevant are experiments with more highly charged redox inactive complexes $[Pt(NH_3)_6]^{4+}$ and $[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$,[73] which are found to associate strongly with plastocyanin (see below). The two stages as in (4) and (5) are now regarded as well established.

Other examples of limiting kinetics have been reported with [2Fe–2S] and 2[4Fe–4S] ferredoxins,[83] with cytochrome b_5[84] and, recently, cytochrome f.[28] Those for the ferredoxins with complexes ranging from 5+ to 2+ are the most complete yet obtained clearly illustrating the effect of charge. The cytochrome f study is of interest because a positively charged locality on a negatively charged protein is involved. Also it is (at present) the only example involving a negatively charged complex and a positively charged locality on a protein (there are no examples of limiting kinetics with stellacyanin or cytochrome c). Earlier studies reporting limiting kinetics which have more recently been questioned include, the oxidation of $[Fe(CN)_6]^{4-}$ with cytochrome c(III),[74] stellacyanin Cu^{I} with $[Co(edta)]^{-}$,[85] azurin Cu^{I} with $[Fe(CN)_6]^{3-}$ (and the reverse),[73] and $[Fe(CN)_6]^{4-}$ with PCu^{II}.[81] A procedure for estimating the local effective charge on a protein surface from association constants has been reported.[86] Results obtained are consistent with structural information.

There are two alternative explanations to (4)—(5), which give rate laws of the same empirical form as (3).[87] One of these is readily dismissed. It involves an initial

[80] S. K. Chapman, I. Sanemasa, A. D. Watson, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1983, 1949.
[81] See comments in *ref.* 80.
[82] R. A. Holwerda, D. B. Knaff, H. B. Gray, J. D. Clemmer, R. Crawley, J. M. Smith, and A. G. Mauk, *J. Am. Chem. Soc.*, 1980, **102**, 1142.
[83] F. A. Armstrong and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **100**, 7710; F. A. Armstrong, R. A. Henderson, and A. G. Sykes, *J. Am. Chem. Soc.*, 1979, **101**, 6912 and 1980, **102**, 6545.
[84] S. K. Chapman, D. M. Davies, C. P. J. Vuik, and A. G. Sykes, *J. Am. Chem. Soc.*, 1984, **106**, 2692.
[85] M. J. Sisley, M. G. Segal, C. S. Stanley, I. K. Adzamli, and A. G. Sykes, *J. Am. Chem. Soc.*, 1983, **105**, 225.
[86] S. K. Chapman, J. D. Sinclair-Day, A. G. Sykes, S.-C. Tam, and R. J. P. Williams, *J. Chem. Soc., Chem. Commun.*, 1983, 1152.
[87] *Ref.* 2, p. 49.

'activation' of the protein, here represented as $P \rightleftharpoons P^*$, followed by reaction of P^* with the redox partner. A stationary-state treatment for P^* gives an equation of the form (3), from which the rate constant for $P \longrightarrow P^*$ can be obtained. Since different values of this rate constant are obtained with different redox partners the mechanism is clearly untenable. The second alternative sometimes described as the 'dead-end' mechanism, is more difficult to dismiss and remains a kinetic ambiguity. This requires (for example) $[Co(phen)_3]^{3+}$ to associate at one binding site to give a completely redox inactive form. The redox reactivity is maintained by the $[Co(phen)_3]^{3+}$ reacting with the remaining protein at an alternative site in a redox process which does not give observable association.

Measurement of proton n.m.r. spectra of PCu^I in the presence of analogue redox inactive Cr^{III} complexes have so far provided no support for conformational changes of the kind required by the dead-end mechanism.[1,16] It seems reasonable therefore that discussion should proceed in terms of (4) and (5) until evidence in support of this alternative is obtained.

13 Information from N.m.r. Studies

The suggestion that different oxidant binding sites are utilised according to charge on the redox partner has been further established by high resolution ^1H n.m.r. spectroscopy. The effect of redox inactive complexes $[Cr(CN)_6]^{3-}$, $[Cr(phen)_3]^{3+}$, and $[Cr(NH_3)_6]^{3+}$ (0.2—1.2 mM) on the n.m.r. of PCu^I (~3.5 mM) has been investigated.[16] Since $[Cr(phen)_3]^{3+}$ ($K = 176\ M^{-1}$) exhibits competitive inhibition for $[Co(phen)_3]^{3+}$ it can be assumed that the two associate at the same site on PCu^I.[75a] The paramagnetic Cr^{III} complexes induce local line-broadening effects and indicate preferred sites for association. Such experiments provide evidence for association of $[Cr(CN)_6]^{3-}$ close to His-87 at the northern hydrophobic patch near to the Cu (which is 6 Å from the surface), whereas $[Cr(phen)_3]^{3+}$ and $[Cr(NH_3)_6]^{3+}$ associate at a site more distant from the Cu and close to Tyr-83. The latter is close to the conserved negatively charged 42—45 and 59—61 regions. Similar behaviour is observed for plastocyanin from three sources, parsley, French bean, and cucumber.[16] Invariance and high conservation of non-polar amino-acid residues close to His-87 has also been noted. The results indicate a high degree of specificity in the binding. Since line broadening is dependent on the inverse sixth-power of the distance of the amino-acid from the paramagnetic centre, the His-87 and Tyr-83 residues are likely to be close to the respective binding sites.

Cytochrome *c*(II) (charge 8+) also induces line broadening of Tyr-83 and evidence for association $K = 1.1 \times 10^3\ M^{-1}$ has been reported with French bean PCu^I.[88] From kinetic studies however, with parsley PCu^{II}[89] there was no curvature of the kind implicit in (3) and it was concluded that $K < 150\ M^{-1}$, $I = 0.10$ M. These results for non-physiological protein reactants of impressively high opposite charges are of considerable interest even though the magnitude of K remains in doubt. A value of $K < 150\ M^{-1}$ indicates considerable mismatch of charge.

[88] G. C. King, R. A. Binstead, and P. E. Wright, *Biochem. Biophys. Acta*, 1985, **806**, 262.
[89] M. A. Augustin, S. K. Chapman, D. M. Davies, A. D. Watson, and A. G. Sykes, *J. Inorg. Biochem.*, 1984, **20**, 281.

14 Effect of pH on Reactivity of PCu^{I}

On decreasing the pH from 7.5, rate constants for the $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidation of PCu^{I} decrease dramatically to zero or a value close to zero.[48] The apparent retention of up to 10% reactivity in some cases[73] at low pHs may be due to the difficulty in determining accurately rate constants at pH < 5. The contribution is in any case small and, if real, does not affect the discussion to follow. The dependence on $[H^+]$ assuming a complete switch-off is summarized by (6),

$$k = \frac{k_0 K_a}{K_a + [H^+]} \tag{6}$$

where the constants are as defined in (7)—(8),

$$H^+PCu^{I} \overset{K_a}{\rightleftharpoons} H^+ + PCu^{I} \tag{7}$$

$$PCu^{I} + \text{oxidant} \xrightarrow{k_0} \text{products} \tag{8}$$

It is now clear that the pK_a from studies with $[Co(phen)_3]^{3+}$ are 0.6—0.8 units higher than those obtained with $[Fe(CN)_6]^{3-}$ as oxidant, Figure 8.[90] An active site pK_a of 4.9 determined some time ago by n.m.r. for spinach PCu^{I} was assigned to protonation and dissociation of His-87.[91] More recently n.m.r. values[78,92] for other plastocyanins have been determined, Table 5. Conditions of ionic strength and temperature show some minor variations. In all cases, however, there is satisfactory agreement with the $[Fe(CN)_6]^{3-}$ kinetic pK_as. Confirmation that protonation and dissociation of His-87 is implicated has come from *X*-ray crystallography.[1] The approach here has been to repeat diffraction measurements and refine calculations for crystals of poplar PCu^{I} prepared at six different pHs from 7.8 to 3.8. The only detectable differences are at the Cu active site. The Cu atom is seen to move away from His-87 and towards Met-92. The Cys-84 side chain follows the Cu atom while the positions of His-37 and Met-92 remain unchanged. At each pH the bond lengths determined are the mean of contributions from the protonated and unprotonated forms. The increase in the Cu–N (His-87) distance to ~3.4 Å is sufficient to accommodate a proton on the imidazole N_δ atom. For energetic reasons oxidation of three-co-ordinate Cu^{I} to Cu^{II} is expected to be difficult.

The higher pK_as for $[Co(phen)_3]^{3+}$ can be accounted for by including a second acid dissociation constant K_a', for a process occurring at the binding site used by $[Co(phen)_3]^{3+}$. Accordingly there are, for positively charged reactants, two protonations one at the active site A and the other at the binding site B. Assuming

[90] J. D. Sinclair-Day, M. J. Sisley, A. G. Sykes, G. C. King, and P. E. Wright, *J. Chem. Soc., Chem. Commun.*, 1985, 505.
[91] J. L. Markley, E. L. Ulrich, S. P. Berg, and D. W. Krogman, *Biochemistry*, 1975, **14**, 4428.
[92] C. L. Kojiro and J. L. Markley, *FEBS Lett.*, 1983, **162**, 54.

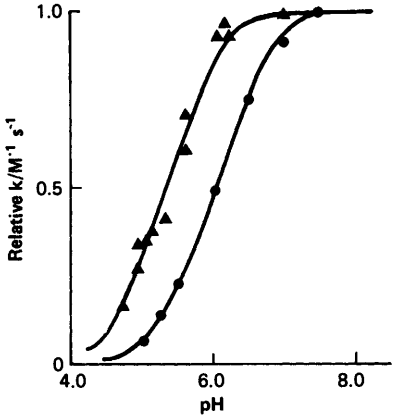

Figure 8 *Variation of rate constants* $k(M^{-1}s^{-1})$ *at* 25 °C *(relative scale) for the oxidation of parsley* PCu^{I} *with* $[Fe(CN)_6]^{3-}$ (▲) *and* $[Co(phen)_3]^{3+}$ (●)

Table 5 *Acid dissociation constants* pK_a *values* (25 °C) *for plastocyanin* PCu^{I} *from different sources as determined by n.m.r. and kinetic studies with* $[Fe(CN)_6]^{3-}$ *and* $[Co(phen)_3]^{3+}$ *respectively as oxidants*

PCu^{I} *Source*	*N.m.r.*	$[Fe(CN)_6]^{3-}$	$[Co(phen)_3]^{3+}$
Parsley	5.7	5.5	6.1
Spinach	4.9	4.9	5.7
French Bean	4.8	4.6	5.4
Anabaena variabilis	5.1	4.8	5.5

the two protonations to be independent the reaction is shown in Scheme 1.[90] Protonation at A gives redox inactive protein. From this scheme the expression (9) is derived.

$$k = \frac{k_1K_aK_a' + k_2K_a'[H^+]}{K_aK_a' + K_a'[H^+] + K_a[H^+] + [H^+]^2} \qquad (9)$$

The experimental data can be fitted to (9) with K_a fixed at the value determined by n.m.r. Figure 9 indicates a best fit for parsley PCu^{I} with $[Co(phen)_3]^{3+}$. Values of pK_a' obtained are for parsley (5.8), spinach (5.6), French bean (5.7) and *A. variabilis* (5.7). For reactions with $[Co(phen)_3]^{3+}$ protonation at B is a major contributing factor, whereas active-site protonation is the predominant and possibly only influence on the $[Fe(CN)_6]^{3-}$ oxidation.

The behaviour observed is consistent with different binding sites for $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, which from the n.m.r. line-broadening studies in the presence of redox inactive Cr^{III} complexes[16] were designated as close to His-87

$$H_2P_{AB} \overset{K_a}{\rightleftharpoons} HP_A \overset{+H^+}{\rightleftharpoons}$$

$$K_a \uparrow\downarrow +H^+ \qquad \uparrow\downarrow K_a$$

$$HP_B \overset{K_a'}{\rightleftharpoons} P + H^+$$

$$P + \text{oxidant} \xrightarrow{k_1} \text{products}$$

$$HP_B + \text{oxidant} \xrightarrow{k_2} \text{products}$$

Scheme 1

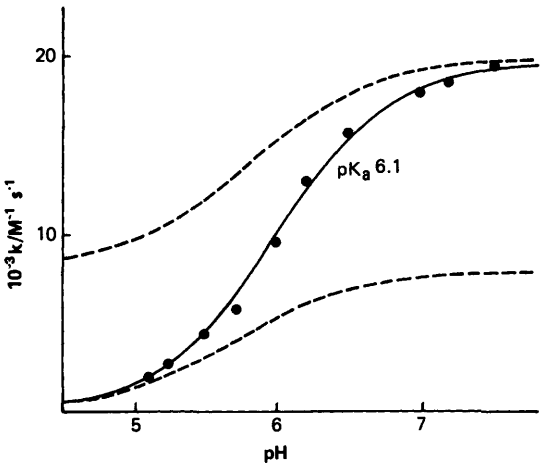

Figure 9 *Variation of rate constant* $k(M^{-1}\ s^{-1})$ *for the oxidation of parsley* PCu^I *by* $[Co(phen)_3]^{3+}$, $I = 0.10\ M$ (NaCl), *with* pH(●). *The broken lines are obtained from the fit of data and indicate the influence of binding site (upper) and active site (lower) protonations. The solid line indicates the overall fit of data to equation* 9

(north) and Tyr-83 (east). Values of pK_a' close to 5.7 are high for protonation at a single carboxylate residue, and could be accounted for by proton sharing between two adjacent carboxylates. There are a number of carboxylates close to Tyr-83, most notably for plant plastocyanins the highly conserved 42—45 patch. Since the 59—61 negative patch is not conserved for parsley plastocyanin this may make a less significant contribution than might previously have been supposed. Protonation of acidic residues will decrease their affinity for positively charged $[Co(phen)_3]^{3+}$ in the association step prior to electron transfer. Also of interest is the response of *A. variabilis* plastocyanin since there are far fewer negatively charged residues, and only 42 of 42—45 is a carboxylate, Figure 3. We note,

however, the close proximity of Glu-85 which like Asp-42 is close to Tyr-83, and could provide an adequate site for association of $[Co(phen)_3]^{3+}$ and for protonation. Relevant distributions of negative charge on the east side of poplar and *A. variabilis* plastocyanin are indicated in Figure 10.

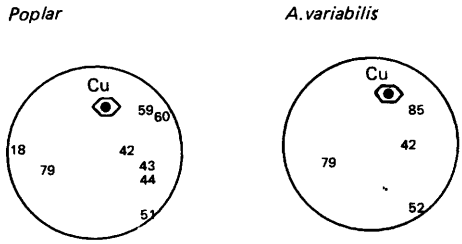

Figure 10 *Distribution of negatively charged carboxylate residues on the east side (normal view) of poplar and* A. variabilis *plastocyanins as seen from the south east of the molecule with the* Cu *and* Tyr-83 *ring aligned*

With regard to the higher pK_as for parsley plastocyanin, sequence information has indicated some striking differences compared to other plastocyanins, most notably the deletion of Met-57 (normally invariant) and residue-58, which are ~10 Å from the active site, and the occurrence of Pro-60 which gives a bend in the peptide chain. Plastocyanin from the algal sources *S. obliquus* and *C. fusca.* are known to have deletions at positions 57 and 58, and will be the subject of a further study in order to understand more fully these effects. Of further interest is the observation that all other single Cu (type-1) proteins investigated including azurin, stellacyanin, umecyanin, and rusticyanin do not display active-site protonation as observed for plastocyanin.[93] Clearly some very delicately balanced mechanism is applicable to give rise to such different behaviour of the plastocyanin Cu^I active site.

15 The Effect of pH on Reactivity of PCu^I

With $[Fe(CN)_6]^{4-}$ as reductant for parsley PCu^{II} the effect of decreasing the pH (7.5 to 4.5) on rate constants is small, Figure 11, and in sharp contrast to behaviour observed for $[Fe(CN)_6]^{3-}$ with PCu^I.[26,48] The implication of these results is that E^0 for the protein increases with decreasing pH as already indicated (Figure 7). Microscopic reversibility requires that $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$ react at the same site (or sites), and that the ratio of rate constants (the redox equilibrium constant) at any one binding site is the same. With $[Ru(NH_3)_5py]^{2+}$,[73] and (presumably) other positively charged reductants yet to be tested, a significant effect of pH is observed, Figure 12. A fit of the kinetic results gives a rate constant k_H for protonated protein which is 36% of k_0 for unprotonated protein (4.2×10^5 M^{-1} s^{-1}), and a pK_a of 4.95. The behaviour observed is consistent with $[Fe(CN)_6]^{4-}$

[93] J. McGinnis, J. D. Sinclair-Day, and A. G. Sykes in 'Biochemical and Inorganic Aspects of Copper Coordination Chemistry', ed. K. D. Karlin and J. Zubieta, Adenine Press, 1985.

reacting at the adjacent north site, which is distant from and only slightly influenced by protonation(s). In contrast $[Ru(NH_3)_5py]^{2+}$ reacts at least partially at the east site which is susceptible to protonation, most likely protonation at the 42—45 patch.

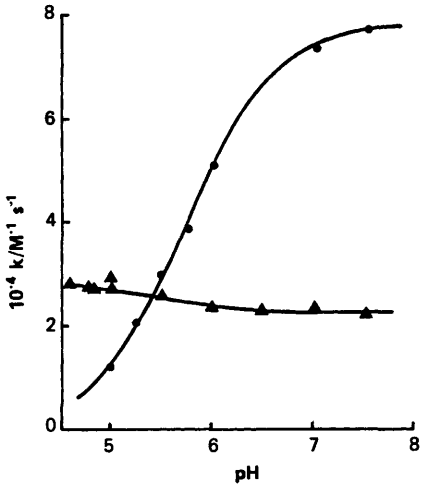

Figure 11 *The variation of rate constants* (25 °C) *for the reactions of parsley plastocyanin* PCu^{I} + $[Fe(CN)_6]^{3-}$ (●), *and* $[Fe(CN)_6]^{4-}$ + PCu^{II} (▲) *with pH, I* = 0.10 M (NaCl)

The above approach has been applied to reactions of PCu^{II} with other proteins, where it is of course essential to have independent information (from studies with inorganic complexes) that pH does not effect the reactivity of the second protein and give rise to ambiguities in interpretation. It has been shown that the reaction of PCu^{II} with the high-potential Fe/S protein (hipip) from *Chromatium vinosum*, charge −3 at pH 7, is independent of pH (5.0—8.5) implicating the adjacent (north) site as binding site.[94] However with cytochrome *c*(II)[94] and cytochrome *f*(II)[28] both give significant pH effects which are more extensive than those observed for $[Ru(NH_3)_5py]^{2+}$. With cytochrome *c*(II) the rate constant for reaction of the protonated form is close to zero, and the pK_a 4.95. With cytochrome *f*(II) (Figure 13) k_H is ~10% of k_0, and the pK_a is 5.07.[28] These findings strongly suggest use of the remote (east) binding site. In the case of cytochrome *c*(II) this is perfectly consistent with the overall charge of 8+ and circle of lysine residues around the exposed haem edge. With regard to cytochrome *f*(II) the position is less certain since the structure is not known. However, in spite of the small overall negative charge (apparent from column behaviour), evidence has accumulated for the presence of a functional positive patch, presumably close to an exposed haem edge on the protein. The sequence has indicated pairs of basic lysine residues at five places in the chain, and it is tempting to speculate that these are brought together in

[94] S. K. Chapman, C. V. Knox, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1984, 2775.

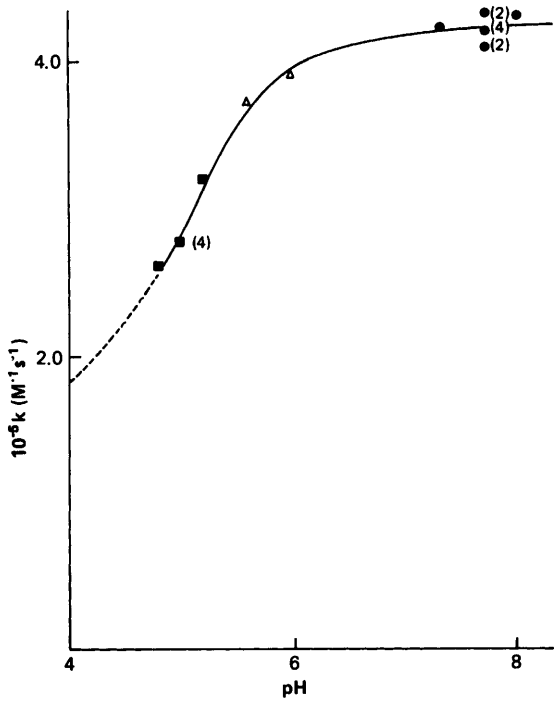

Figure 12 *The effect of pH on rate constants* (25 °C) *for the* $[Ru(NH_3)_5py]^{2+}$ *reduction of* PCu^{II} *at* I = 0.10 M (NaCl); *buffers–acetate* (■), *mes* (△) *and Tris/maleate* (●)

the tertiary structure to form a positive patch capable of interacting with the negative patch on plastocyanin, its natural partner.[11]

The pK_as of around 5.0 observed in the above studies involving PCu^{II} are in contrast to values (pK_a') of ~5.7 obtained for protonation at the binding site in the $[Co(phen)_3]^{3+}$ oxidation of PCu^{I}. It is puzzling that similar values are not observed since the Cu charge would not have been expected to be influential at the distance of the Tyr-83 binding site, even though the connecting peptide is conserved. Using near-u.v., fluorescence, and c.d. measurements, plastocyanin from higher plants including spinach and poplar has been shown to undergo conformational changes upon reduction and lowering of the pH.[41] Fluorescence technique is particularly effective since plastocyanin contains only a few tyrosines (three for spinach and two for poplar) and no tryptophans. Each of the tyrosines is located in a different region making it possible to determine which parts of the molecule undergo conformational changes. In addition Tyr-83, which is exposed to solvent, has been chemically modified to nitrotyrosine which is non-fluorescent. The results obtained show that the east face of the molecule which incorporates Tyr-83 and Tyr-80

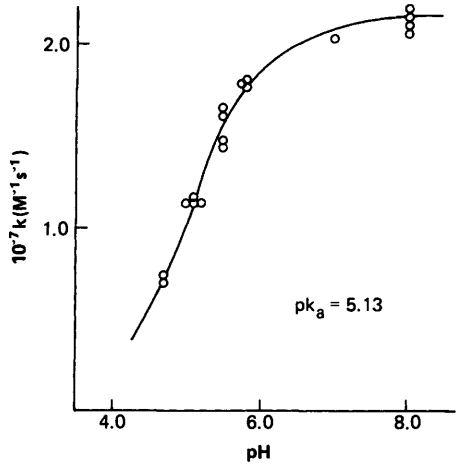

Figure 13 *The effect of pH on the cytochrome f*(II) *reduction of parsley* PCu^{II} *at* 25 °C, I = 0.10 M

undergoes conformational change upon reduction, and that the effects observed are pH dependent. These results contrast with the crystallographic information which indicates nearly identical PCu^{I} and PCu^{II} structures at neutral pH.[1] The solution effects observed are very much lessened by addition of 100 mM $CaCl_2$ or 2.7 M $(NH_4)_2SO_4$, and an explanation may be related to the fact that crystallization was from 2.7 M $(NH_4)_2SO_4$. Conformational changes could explain the different pK_as for PCu^{I} (5.7) and PCu^{II} (5.0) which have been assigned to protonation at the east site, and for the simplest most consistent explanation involve the same residue. In the wider context the effects observed are important in so far as the natural function of the protein is concerned, since the affinity of cytochrome *f*(II) and PCu^{II} for each other prior to electron transfer has to be reconciled with less affinity required after electron transfer, so that the proteins separate efficiently. In other words the conformational changes identified could well promote differential binding of the oxidized and reduced forms of plastocyanin to the redox partner.

Further information is required, and in particular studies on *A. variabilis* plastocyanin, which are in progress, could be extremely helpful.

16 Competitive Inhibition

This is observed in the $[Co(phen)_3]^{3+}$ oxidation of PCu^{I} in the presence of redox inactive complexes $[Cr(phen)_3]^{3+}$, $[Co(NH_3)_6]^{3+}$, $[Pt(NH_3)_6]^{4+}$, and $[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$. The effect stems from competitive association at the binding site (or sites) used by $[Co(phen)_3]^{3+}$. Inhibition by $[Cr(phen)_3]^{3+}$ is an important link with n.m.r. experiments already referred to. Blocking with the 3+ complexes is not sufficiently extensive, however, to indicate clearly the effectiveness

at high 3+ concentrations. With the 4+ and 5+ complexes on the other hand (here designated B), it is clearly established that blocking at high concentrations is incomplete, Figure 14. The extent of the effect (recently verified [77]) appears to be independent of the size and charge of B and for both 4+ and 5+ complexes the adduct PCu^{I}, B retains 50% reactivity with $[Co(phen)_3]^{3+}$ at pH 5.8. Interestingly, a similar influence of protonation is observed as with inhibition by metal complexes. At relatively low $[Co(phen)_3]^{3+}$, when $K[Co(phen)_3{}^{3+}]$ as in equation 3 can be neglected, the reactions (10)—(12),

$$PCu^{I} + Co^{III} \xrightarrow{Kk_{et}} \text{products} \tag{10}$$

$$PCu^{I} + B \rightleftharpoons PCu^{I}, B \quad (K_B) \tag{11}$$

$$PCu^{I}, B + Co^{III} \xrightarrow{k_B} \text{products} \tag{12}$$

$$k_{exp} = \frac{Kk_{et} + k_B K_B[B]}{1 + K_B[B]} \tag{13}$$

gives a dependence shown in equation 13, and the variation of second-order rate constants k_{exp} with (B) can be studied.

Values of association constants K (and K_B) in Table 6, are consistent with charge being important. To avoid acid dissociation of $[Pt(NH_3)_6]^{4+}$ (pK_a 7.1) with formation of a 3+ complex, a pH of 5.8 is adopted to give maximum effectiveness. There is no similar problem with the 5+ complex which remains undissociated to

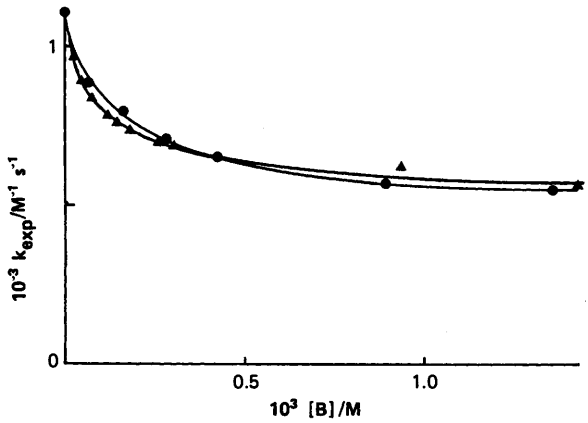

Figure 14 *The blocking effect of redox-inactive* $[Pt(NH_3)_6]^{4+}$ *and* $[(NH_3)_5CoNH_2 \cdot Co(NH_3)_5]^{5+}$ *on the* $[Co(phen)_3]^{3+}$ *oxidation of parsley plastocyanin,* PCu^{I}. *Rate constants determined at* 25 °C, *pH* 5.8, I = 0.10 M (NaCl)

higher pHs, but for comparison it is sometimes convenient, as in Figure 13, to work also at pH 5.8. Consistent with the electrostatics the 4+ and 5+ complexes give the biggest association constants. Because of its spherical symmetry and high charge density (as well as ease of preparation), $[Pt(NH_3)_6]^{4+}$ is probably the most appropriate inhibitor to select.

Table 6 *Association constants (K) for inorganic complexes with parsley plastocyanin,* PCu^I, *at* 25 °C, I = 0.10 M (NaCl)

Complex	*pH*	K/M^{-1}
$[Co(phen)_3]^{3+}$	7.5	167
$[Cr(phen)_3]^{3+}$	7.5	176
$[Co(NH_3)_6]^{3+}$	7.5	580
$[Pt(NH_3)_6]^{4+}$	5.8	1.6×10^{4} [a]
$[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$	5.8	0.8×10^{4} [a,b]

[a] From data in Figure 13. [b] 1.6×10^4 M^{-1} at pH 7.5

It has been observed in other related studies of the [2Fe–2S] and 2[4Fe–4S] ferredoxins with inorganic complexes that there is complete blocking by redox-inactive complexes such as $[Cr(en)_3]^{3+}$. An important question is raised therefore in the case of plastocyanin by the observation of only partial inhibition *i.e* k_B is not zero. There are two possible explanations. The first is that because the negative charge in the vicinity of Tyr-83 of plastocyanin is widely distributed it is possible to retain some reactivity of the PCu^I, B adduct in equation 13. This requires that two positively charged complexes can be accommodated simultaneously at the east site. The second is that reaction corresponding to k_B is at the adjacent north site, even though in the n.m.r. experiments $[Cr(phen)_3]^{3+}$ line broadening of the His-87 resonances was not observed. As long as there were two prominent negative patches 42—45 and 59—61 either side of Tyr-83 the first of these remained a strong possibility. Now that the sequence of parsley has been determined and it has been demonstrated that 59—61 is no longer invariant (and is only 1−), this assignment is perhaps less likely, particularly as rate constants and the reactivity pattern for parsley plastocyanin is so similar to that for other higher plant plastocyanins.

For these reasons the reaction of $[Co(phen)_3]^{3+}$ with parsley PCu^I may occur 50% (in the case of parsley plastocyanin) at the north site, with the rest of the reaction at the east. Whether for this to hold it is reasonable that a single proton should completely switch off reactivity in a locality (B) of considerable negative charge is a key question. Evidence for a conformational change has been presented from fluorescence experiments, and this might help provide a satisfactory explanation. It has been found that K_B for association of $[Pt(NH_3)_6]^{4+}$ with PCu^I decreases to zero as the pH is decreased.[73] Experiments aimed at determining the effect of pH on K and k_{et} for $[Co(phen)_3]^{3+}$ oxidation of PCu^I are difficult due to the smallness of K. The effect of protonation on the $[Ru(NH_3)_5py]^{2+}$ reduction of PCu^{II} can likewise be explained by reaction at both the north and east sites.

Both the cytochrome c(II)[94] and cytochrome f(II)[28] reductions of PCu^{II} are substantially inhibited by $[Pt(NH_3)_6]^{4+}$. At pH 5.8 the former gives a (maximum)

73% blocking effect. The rapidity of the cytochrome *f*(II) reaction necessitated the reaction being carried out at 10 °C, $I = 0.20$ M (NaCl). Under these conditions complete blocking appears to hold, Figure 15, with $K_B = 1\,600$ M^{-1}. As was concluded also from $[H^+]$ effects, both cytochromes have much greater specificity for the east site. Complementary matching surfaces in the case of cytochrome *f* and plastocyanin would be expected to give higher specificity, with comparatively large surface contact areas.

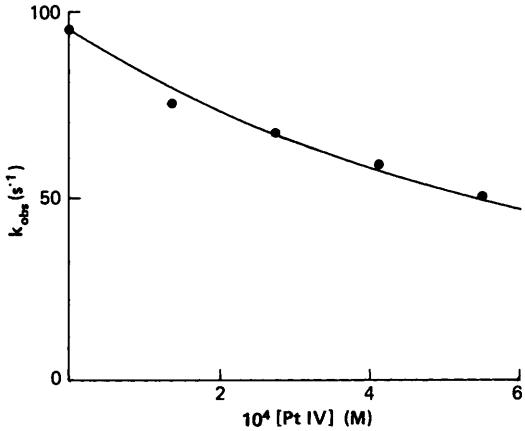

Figure 15 *The effect of redox-inactive* $[Pt(NH_3)_6]^{4+}$ *on the cytochrome* f(II) *reduction of parsley* PCu^{II} *at* 10 °C, *pH* 5.8, $I = 0.20$ M (NaCl)

Since limiting kinetics are not observed in the $[Co(phen)_3]^{3+}$ oxidation of *A. variabilis* PCu^{I} it is hardly surprising that $[Pt(NH_3)_6]^{4+}$ does not inhibit the reaction. Analysis of the effect of pH on rate constants has indicated that here also ~50% of reaction is at the east site (pK_a 5.7). There is no effect of $[Pt(NH_3)_6]^{4+}$ on the $[Co(dipic)_2]^-$ oxidation of parsley PCu^{I} consistent with reaction at the north site.[73] The increase in rates for the $[Fe(CN)_6]^{4-}$ and Hipip(r) reduction of PCu^{II} in the presence of $[Pt(NH_3)_6]^{4+}$ can be explained by these reactants using the east as well as the north sites in the presence of associated $[Pt(NH_3)_6]^{4+}$, and/or reactant ion pairs such as $[Pt(NH_3)_6]^{4+}$, $[Fe(CN)_6]^{4-}$ exhibiting different behaviour.[73,94]

17 Electron-exchange Rate Constants

In a recent study of the line-broadening observed at 50 °C in the proton n.m.r. spectrum of a partly oxidized solution of *Pseudomonas aeruginosa* azurin an electron self-exchange rate constant of 2×10^6 M^{-1} s^{-1} has been reported.[95] A variety of values from 2.8×10^{-2} to 8×10^7 M^{-1} s^{-1} have been obtained using the Marcus treatment for a range of redox partners.[96,97] The value obtained compares

[95] G. W. Canters, H. A. O. Hill, N. A. Kitchen, and E. T. Adman, *J. Magn. Reson.*, 1984, **57**, 1.
[96] D. Cummins and H. B. Gray, *J. Am. Chem. Soc.*, 1980, **102**, 4360.
[97] A. G. Mauk, R. A. Scott, and H. B. Gray, *J. Am. Chem. Soc.*, 1980, **102**, 4360.

well with 9×10^5 M^{-1} s^{-1} at 20 °C obtained by Wherland and Pecht from studies on protein–protein reactions.[23] An earlier value reported for bean plastocyanin is $\ll 2 \times 10^4$ M^{-1} s^{-1} [98] If indeed the exchange of plastocyanin is so slow then it is tempting to relate the difference to the different charge distributions over the protein surfaces. The existence of negatively charged regions on plastocyanin has been construed as evidence for the importance of electrostatic interactions in the reaction with other redox partners. The repulsive effect of two such regions could provide an explanation of the slow self-exchange, although exchange *via* two hydrophobic north sites would remain a reasonable alternative.

A preliminary value of the self-exchange rate constant for stellacyanin of 1×10^5 M^{-1} s^{-1} using an e.p.r. method[99] compares with values previously reported using the Marcus treatment from 6×10^3 M^{-1} s^{-1} for electron transfer with *Pseudomonas aeruginosa* cytochrome c_{551}, to 2.9×10^4 M^{-1} s^{-1} with cytochrome *c*, 1.7×10^5 M^{-1} s^{-1} with $[Ru(NH_3)_5py]^{3+}$, 2.3×10^5 M^{-1} s^{-1} with $Fe(edta)]^{2-}$, and 3.0×10^5 M^{-1} s^{-1} with $[Co(phen)_3]^{3+}$

18 Modifications

Reduction of French bean PCu^{II} by hexa-aqua Cr^{2+} gives a product which it has been demonstrated has Cr^{III} attached.[100,101] Since there is no binding of a solution of hexa-aqua Cr^{III} with PCu^{I} at pH ~5 the reaction falls within the classification of inner-sphere electron transfer. From thermolysin proteolysis experiments on the product obtained at pH 7 using labelled Cr^{2+}, it has been concluded that the label is contained in the peptide fraction containing amino-acid residues 40—49.[101] Co-ordination of the Cr at one or two carboxylates in the 42—45 patch is favoured. The Cr^{III} modified protein exhibits reactivity consistent with modification at the 42—45 patch, with a net slowing down of the reaction with positively charged redox partners such as $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5py]^{2+}$,[102] as well as cytochrome *c*[94] and cytochrome *f*,[28] but no effect is observed on the reaction with $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^{-}$, and Hipip Fe/S protein.[28,94] However, there are features which cast some doubt on the whole approach. Since $[Cr(H_2O)_6]^{3+}$ has a pK_a of 3.8, attachment of an aqua Cr^{III} moiety to a protein at pH ~7 must be viewed with some concern. Formation of Cr^{III} conjugate-base forms with a resultant labilizing effect on the normally inert co-ordination sphere could mean that the Cr^{III} does not remain attached at the point of Cr^{2+} attack. Experiments at pH 5.8 moreover indicate the presence of at least two different Cr^{III}-modified forms are present. Other Cr^{II} complexes having protective ligands might usefully be explored here. Unfortunately the Cr^{II} complex of the tetradentate macrocyclic ligand 15aneN$_4$ (1,4,8,12-tetra-azacyclopentade-

[98] J. K. Beattie, D. J. Fenson, H. C. Freeman, E. Woodcock, H. A. O. Hill, and A. N. Stokes, *Biochim. Biophys. Acta*, 1975, **405**, 109.
[99] S. Dahlin, B. Reinhammar, and M. T. Wilson, *Inorg. Chim. Acta*, 1983, **79**, 126.
[100] O. Farver and I. Pecht, *Biochemistry*, 1982, **21**, 1885.
[101] O. Farver, Y. Shahak, and I. Pecht, *Biochemistry*, 1982, **21**, 1885.
[102] S. K. Chapman, C. V. Knox, P. Kathirgamanathan, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1984, 2769.

cane), $[Cr(15aneN_4)(H_2O)_2]^{2+}$, which reacts inner-sphere with [2Fe–2S] and 2[4Fe–4S] ferredoxin,[103] reacts by an outer-sphere mechanism with PCu^{II}.

Spinach plastocyanin has been chemically modified using a water soluble carbodi-imide to form an amide bond between a protein carboxyl group and an amine group of ethylenediamine, equation 15.[104]

$$\text{protein-}CO_2^- + NH_2C_2H_4NH_3^+ \longrightarrow \text{protein-}CONHC_2H_4NH_3^+ \qquad (15)$$

Four distinct chemically modified products containing 2.1, 3.2, 4.1, and 6.3 mol of ethylenediamine per mol of plastocyanin were separated by gel electrophoresis and ion-exchange chromatography.[105] The location of the modified residues was determined for the fraction containing 3.1 mol of ethylenediamine. A two-fold enrichment of the ^{14}C-labelled ethylenediamine was found in the tryptic peptide containing residues 31—55. The modifications give an increase in reduction potential of +40 mV.

The effect of ethylenediamine modification of plastocyanin on electron donation by plastocyanin to $P700^+$ in photosystem I particles has been studied.[106] The reduction of such particles by unmodified plastocyanin requires the presence of divalent cations such as Mg^{2+} to screen the negative charges on the two reactants and hence facilitate interaction.[107] Although the chemically modified plastocyanins bind more strongly with PSI, it has been concluded that the 42—45 region is probably not the binding site for $P700^+$.[105] The effect of cytochrome *f* modifications on reactivity with plastocyanin are also being explored.[108]

Evidence for cytochrome *f*(II) reacting at the remote Tyr-83 site of PCu^{II} has been obtained from the effects of pH, $[Pt(NH_3)_6]^{4+}$, and Cr^{III} modification.[28] The occurrence of the haem-containing globular part of cytochrome *f* (Figure 16), and plastocyanin in the inner thylakoid suggests that the two are able to orientate and dock in an electrostatically controlled process. It is often assumed that because two binding sites have been identified for isolated plastocyanin, that the same two sites must be biologically relevant, the one in the reaction with cytochrome *f* and the other with $P700^+$. This is possible and even likely, but has yet to be established. The hydrophobic north surface may alternatively serve solely to orientate plastocyanin by associating favourably at the thylakoid surface.

A further type of modification should be mentioned, which is providing much additional important information. The groups of Gray and Isied have attached Ru^{III} to metalloproteins so that fixed-site long-distance electron transfer between metal centres separated by distances >10 Å can be studied. Two examples have so far been reported and others are being investigated. The approach is to attach

[103] I. K. Adzamli, R. A. Henderson, J. D. Sinclair-Day, and A. G. Sykes, *Inorg. Chem.*, 1984, **23**, 3069.
[104] K. O. Burkey and E. L. Gross, *Biochemistry*, 1981, **20**, 5495.
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[106] T. Takabe, H. Ishikawa, and S. Niwa, *J. Biochem.*, 1983, **94**, 1901.
[107] T. Takabe, H. Ishikawa, and S. Niwa, *J. Biochem.*, 1984, **96**, 1813.
[108] K. Takenata and T. Takabe, *J. Biochem.*, 1984, **96**, 1813.

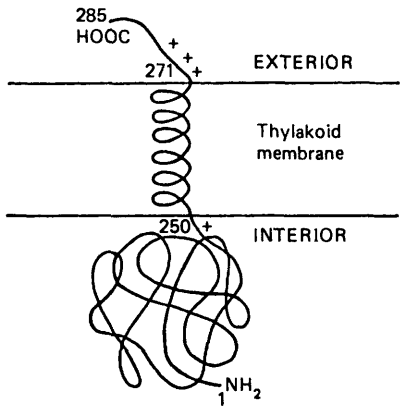

Figure 16 *Schematic representation of the trans-membrane orientation of cytochrome* f *based on the occurrence of the extended stretch of hydrophobic residues* 250—271, *and the high* (22%) *charge composition of the* 1—250 *section* (Ref. 11)

$(NH_3)_5Ru^{II}$ to the His-35 of cytochrome *c*,[109,110] and the His-83 of azurin.[111,112] The Ru^{III} of the fully oxidized adduct is then reduced *in situ* by flash photolysis[109] or pulse radiolysis,[110] when the slow subsequent (first-order) intramolecular electron transfer between the Ru^{II} and Fe^{III} (30 s^{-1})[109] or Cu^{II} (1.6 s^{-1})[112] is observed over distances of ~12 Å defined in crystallographic studies. Only by extending the range of such studies will it be possible to explore fully the relationship of rate constants to distances, and comment more meaningfully on the relative merits of direct as opposed to through atom (*via* the peptide chain) electron transfer (both of which may be relevant), and comment on the part which intervening groups such as aromatic residues play. In this same context molecules containing an electron donor and electron acceptor linked by a rigid non-conjugate bridge are being studied to obtain relevant information.[113–117] Many of these points were addressed by Gray in his recent Centenary Lecture. In the experiments with Ru-modified proteins the sites of attachment are not the binding sites relevant to biological electron-transfer, but sites determined by the presence of histidine residues suitable for modification. In future work there is no

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[110] S. S. Isied, C. Kuehn, and G. Worosila, *J. Am. Chem. Soc.*, 1984, **106**, 1722.
[111] N. M. Kostič, R. Margalit, C.-M. Che, and H. B. Gray *J. Am. Chem. Soc.*, 1983, **105**, 7765.
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[115] C. A. Stein, N. A. Lewis, and G. Seitz, *J. Am. Chem. Soc.*, 1982, **104**, 2596.
[116] D. N. Beratan and J. J. Hopfield, *J. Am. Chem. Soc.*, 1984, **106**, 1584.
[117] N. S. Hush, M. N. Padon-Row, E. Cotsaris, H. Oevering, J. W. Verhoeven, and M. Heppener, *Chem. Phys. Lett.* in press.

reason why the carboxylates of the 42—45 patch should not be modified by attachment of metals. It is also worth noting that in plastocyanin from the algae *S. obliquus* and *A. variabilis* residue 59 is a histidine which is close to Tyr-83 and of interest therefore as a centre for modification.

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