The Reactivity of Spinach Plastocyanin Mutants with Inorganic Oxidants $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$

Panayotis Kyritsis,[a] Lennart G. Lundberg,[b] Margareta Nordling,[b] Tore Vänngård,[b] Simon Young,[b] Nicholas P. Tomkinson[a] and A. Geoffrey Sykes[a]

[a] *Department of Chemistry, The University, Newcastle upon Tyne NE1 7RU, UK*
[b] *Department of Biochemistry and Biophysics, Chalmers University of Technology and University of Göteborg, S-41296, Göteborg, Sweden*

Stopped-flow rate constants for the oxidation of spinach plastocyanin PCu^{I} mutants Leu12Glu, Leu12Asn, Asp42Asn and Tyr83Phe have been determined, and are discussed in terms of two-site reactivity for electron transfer.

Plastocyanin (M_r 10500; 97–99 amino acids) is a single copper (type 1) protein which is a component of photosynthetic electron transport. Extensive sequence[1] and structural information[2] is available and recent reviews have appeared.[1,3] A prime feature of reactivity patterns to date is the identification of adjacent (to the Cu) and remote sites on the surface for electron transfer. The remote site has substantial negative charge which is believed to be important in terms of recognition, making this an attractive possibility for electron transfer with cationic reactants. It has not been possible to define the precise region of surface involved, and indeed fairly non-specific areas may be relevant. In the case of spinach plastocyanin for example the remote patch is regarded as encompassing the seven negatively charged residues 42–45 and 59–61 either side of the conserved Tyr83. It has been noted that electron transfer between the Cu *via* Cys84 and Tyr83 at the remote site is very similar to the Cys *via* His intramolecular electron-transfer path from the type 1 Cu to the type 3 Cu's in the multicopper enzyme ascorbate oxidase.[4] Azurin, on the other hand, may react largely *via* the adjacent hydrophobic patch, which is similarly structured to that of plastocyanin.[5] In this case there is no negatively charged region.[6]

The recently reported expression of spinach plastocyanin in *Escherichia coli*,[7] has led to the successful isolation of a number of singly modified variants by site directed mutagenesis. We have commenced a programme of study in which the reactivity of a series of carefully selected mutants is being explored, and here report findings for four of these, two of which have changes at Leu12 at or near to the adjacent site and two with changes at Asp42 and Tyr83 respectively at the remote site, (Fig. 1). Purification of mutants[8] was by Pharmacia FPLC Mon-Q anion-exchange, and the purity confirmed by SDS-PAGE analysis. The UV–VIS absorbance (A) ratio $A_{278}:A_{597} = 1.2$.

Stopped-flow rate constants have been determined by monitoring the PCu^{I} to PCu^{II} absorbance increase at 597 nm ($\Delta\epsilon$ 4500 $M^{-1}cm^{-1}$).† Protein concentrations were $\sim 2 \times 10^{-5}$ M

† Non SI units mol dm^{-3} = M used.

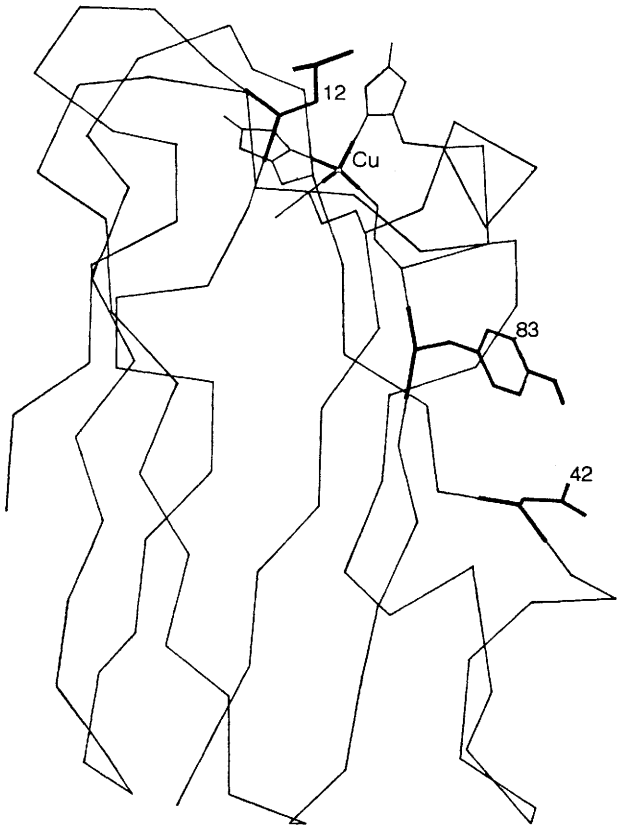

Fig. 1 Structure of plastocyanin from ref. 2, with the positions of residues which are the site of mutations indicated in bold

and the inorganic oxidant was in >tenfold excess. Rate constants obtained for a sample of wild-type spinach plastocyanin obtained by *E. coli* expression are *ca.* 15% different from results reported previously for native spinach plastocyanin.[9] The pH, 10 mM Tris–HCl buffer, was varied over the range 7.0–8.0, $I = 0.100$ M (NaCl), and results listed in Table 1 are for pH 7.5.

Reduction potentials for the PCu^{II}/PCu^{I} couple [*vs.* normal hydrogen electrode (NHE)] determined by equilibration against $[Fe(CN)_6]^{3-/4-}$ show little variation for wild type and mutant forms (± 10 mV) in agreement with previously determined values for the native protein, (Table 1).

For higher plant plastocyanins there is now wide acceptance of the view that the oxidant $[Fe(CN)_6]^{3-}$ reacts at the adjacent site which is non-polar/hydrophobic. Line broadening NMR studies[9] on PCu^{I} in the presence of redox inactive $[Cr(CN)_6]^{3-}$ as well as kinetic measurements[3], have provided substantial support for this assignment.[10] From X-ray crystallography,[2] the Cu is ~6 Å buried at this site which is the closest the Cu approaches to the surface. The invariant Leu12 residue[1] is nearby and is identified as an appropriate residue for site directed mutagenesis. The Leu12Glu change, which at pH 7.5 introduces a 1− charge, results in a sevenfold decrease in rate constant for the reaction with $[Fe(CN)_6]^{3-}$, consistent with the earlier assignment. We are at present less confident in explaining the fivefold increase in rate constant for the Leu12Asn mutant with the same reactant. Understandably the Asp42Asn and Tyr83Phe changes at or near to the remote site have little or no effect on the reaction with $[Fe(CN)_6]^{3-}$.

In the case of cationic inorganic complexes NMR studies with redox inactive $[Cr(phen)_3]^{3+}$ and $[Cr(NH_3)_6]^{3+}$ have indicated a favourable interaction at the remote site.[9] Again

Table 1 Rate constants at 25 °C/M^{-1} s^{-1} for the $[Fe(CN)_6]^{3-}$ (k_{Fe}) and $[Co(phen)_3]^{3+}$ (k_{Co}] oxidation of spinach PCu^{I} native wild type and mutant forms at pH 7.5, $I = 0.100$ M (NaCl)

Protein	$E°$/mV	$10^5 k_{Fe}$	$10^3 k_{Co}$	k_{Fe}/k_{Co}
Native	375	0.85	2.54	33.4
Wild-type	374	0.71	2.24	31.7
Leu12Glu	360	0.11	4.8	2.3
Leu12Asn	365	3.7	2.50	148
Asp42Asn	374	0.85	2.24	37.5
Tyr83Phe	374	0.71	2.64	26.9

substantial support has been obtained from kinetic studies with positively charged inorganic reactants.[1] Using redox inactive inhibitors of high positive charge it has been demonstrated that $[Co(phen)_3]^{3+}$ reacts 75 : 25 at the remote and adjacent sites, respectively.[3] With protein reactants such as cytochrome c and cytochrome f there appears to be an even higher specificity for the remote site.[11] Here we have tested the reaction of four mutants with $[Co(phen)_3]^{3+}$. The most striking observation is the enhancement in rate constant with the Leu12Glu mutant which is understandable in terms of a greater proportion of reaction occurring at the adjacent site due to the more favourable electrostatics. The change Tyr83Phe with retention of the aromatic residue has no effect on reactivity. However, what is most surprising is the observation that the Asp42Asn change at the remote site has little or no effect. This and other mutants are being further investigated.

In addition we have successfully singly Ru-modified and characterised the Tyr83His mutant [Ru : Cu metal analysis by inductively coupled plasma (ICP)] with a view to determining the rate constants for intramolecular $Ru^{II} \rightarrow Cu^{II}$ electron transfer.

We thank the MRI of the SERC (N. P. T.), the State Scholarship Foundation of Greece (P. K.) and the Swedish Natural Science Research Council for financial support .

Received, 27th June 1991; Com. 1/03200C

References

1 See *e.g.* summary in A. G. Sykes, *Adv. Inorg. Chem.*, 1991, **36**, 381.
2 J. M. Guss and H. C. Freeman, *J. Mol. Biol.*, 1983, **169**, 521; J. M. Guss, P. R. Harrowell, M. Murata, V. A. Norris and H. C. Freeman, *J. Mol. Biol.*, 1986, **192**, 361.
3 A. G. Sykes, *Struct. Bonding*, 1991, **75**, 175-224.
4 A. Messerschmidt, A. Rossi, R. Ladenstein, R. Huber, M. Bolognesi, G. Gatti, A. Marchesini, T. Petruzzelli and A. Finazzi-Agro, *J. Mol. Biol.*, 1989, **206**, 513.
5 M. Van de Kamp, R. Floris, F. C. Hali and G. W. Canters, *J. Am. Chem. Soc.*, 1990, **112**, 907.
6 G. E. Norris, B. F. Anderson and E. N. Baker, *J. Mol. Biol.*, 1983, **165**, 504, and *J. Am. Chem. Soc.*, 1986, **108**, 2784; E. N. Baker, *J. Mol. Biol.*, 1988, **203**, 1071.
7 M. Nordling, T. Olausson and L. G. Lundberg, *FEBS Lett.*, 1990, **276**, 98.
8 M. Nordling, K. Sigfridsson, S. Young, L. G. Lundberg and O. Hansson, *FEBS Lett.*, in the press.
9 J. D. Sinclair-Day and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1986, 2069.
10 D. J. Cookson, M. T. Hayes and P. E. Wright, *Biochim. Biophys. Acta*, 1980, **591**, 162; P. M. Handford, H. A. O. Hill, R. W.-K. Lee, R. A. Henderson and A. G. Sykes, *J. Inorg. Biochem.*, 1980, **13**, 83.
11 D. Beoku-Betts, S. K. Chapman, C. V. Knox and A. G. Sykes, *Inorg. Chem.*, 1985, **24**, 1677; M. A. Augustin, S. K. Chapman, D. M. Davies, A. D. Watson and A. G. Sykes, *J. Inorg. Biochem.*, 1984, **20**, 281; V. A. Roberta, H. C. Freeman, A. J. Olson, J. A. Trainer and E. D. Getzoff, *J. Biol. Chem.*, 1991, **266**, 13431.