A New Approach to the Study of Intramolecular Electron-Transfer Reactions of Metalloproteins: Pulse Radiolysis of NO_2-Modified Tyrosine Derivatives of Plastocyanin

K. Govindaraju, Hans E. M. Christensen,† Emma Lloyd, Marianne Olsen,‡ G. Arthur Salmon,§ Nicholas P. Tomkinson, and A. Geoffrey Sykes*

Department of Chemistry, The University, Newcastle upon Tyne NE1 7RU, England

Received July 2, 1991

Single NO_2-modified derivatives of plastocyanin, with modifications at Tyr83 (spinach) and Tyr83/Tyr62 (parsley, deletions at 57 and 58), have been prepared and characterized by peptide mapping. All three products give a $TyrNO_2$ UV-vis absorbance band at 355 nm (ϵ = 3900 M^{-1} cm^{-1}) at pH <7.5, assigned to NO_2, which shifts to 428 nm on increasing the pH to >9.0 due to acid dissociation of the phenolic group. Pulse radiolysis experiments in which the reductant $CO_2^{\bullet-}$ (or Cd^+) is generated give with all three $PCu^{II}TyrNO_2$ derivatives one observable reaction step in which PCu^{II} is reduced to PCu^{I}. Rate constants are of order of magnitude 10^8 M^{-1} s^{-1} ($CO_2^{\bullet-}$) and 10^9 M^{-1} s^{-1} (Cd^+). No $PCu^{II}TyrNO_2^{\bullet-}$ radical intermediate absorbing at ~300 nm ($\epsilon \sim 10^4$ M^{-1} cm^{-1}) is observed. However in studies with $PCu^{I}TyrNO_2$, reduction of the NO_2 group is the only reaction step, with rate constants of the order of magnitude 10^9 M^{-1} s^{-1} (Cd^+), and $PCu^{I}TyrNO_2^{\bullet-}$ is a relatively long-lived product. There is no crystallographic or spectroscopic evidence to suggest any Cu interaction with the phenolate or vice versa (separation >8.6 Å). If this is the case, it can be assumed that $TyrNO_2^{\bullet-}$ is generated at about the same rate in experiments on $PCu^{II}TyrNO_2$ and that rapid $>10^7$ s^{-1} intramolecular electron transfer $PCu^{II}TyrNO_2^{\bullet-} \rightarrow PCu^{I}TyrNO_2$ follows. We note that the rate constant for formation of $PCu^{I}TyrNO_2^{\bullet-}$ is of the same magnitude as the difference in rate constants for $CO_2^{\bullet-}$ (or Cd^+) with native PCu^{II} and $PCu^{II}TyrNO_2$. Also the driving force for $PCu^{II}TyrNO_2^{\bullet-} \rightarrow PCu^{I}TyrNO_2$ of ~800 mV (0.8 eV) favors rapid intramolecular electron transfer. The Beratan-Onuchic pathway program has been used to examine the best electron-transfer pathways from the Tyr83 and Tyr62 derivatives, and favorable through-bond pathways have been identified.

Introduction

Plastocyanin is a single (type 1) blue Cu protein involved in photosynthetic electron transport in the chloroplasts of higher plant and algal species.[1] The tyrosine NO_2 modifications of plastocyanin described herein, in combination with pulse radiolysis studies to give $TyrNO_2^{\bullet-}$, define a new approach in the study of intramolecular electron-transfer ET processes.[2-5] Whereas spinach plastocyanin (M_r = 10 500; 99 amino acids) gives only one product modified at Tyr83, parsley plastocyanin (97 amino acids, including a tyrosine at 62) gives two products with modifications at Tyr83 and Tyr62, respectively. Two regions on the surface of plastocyanin have been identified as relevant to bimolecular ET.[1] One of these, the remote acid patch, has negatively charged residues at 42-45 and 59-61 either side of Tyr83 and is used by positively charged inorganic and metalloprotein reactants. Negatively charged reagents on the other hand prefer the adjacent (to the Cu) His87 site.[1] The tyrosine incorporated at 62 when there are deletions at 57 and 58 may be relevant also in considering ET from the remote site.

Previously, in studies on (nitrophenolato)pentaamminecobalt(III) complexes of the kind $[Co(NH_3)_5O_2C-X-PhNO_2]^{2+}$, where X = CH_2, CH=CH, and other groups of varying complexity, Hoffman and colleagues[6] have demonstrated that the NO_2 moiety can be reduced to $NO_2^{\bullet-}$ (which absorbs at ~300 nm) by reduction with e_{aq}^-, $CO_2^{\bullet-}$, or $Me_2\dot{C}OH$ using pulse radiolysis techniques. Subsequently intramolecular ET from the $NO_2^{\bullet-}$ radical to Co(III) is observed. Rate constants for these mainly nonrigid systems, in which the distance separating $NO_2^{\bullet-}$ and Co(III) is uncertain, and for a related μ-nitrobenzoato Co^{III}_2 complex,[7] are in the range 1×10^5 s^{-1}. The reduction potential of the phenolate $NO_2^{\bullet-}$ radical has been determined as ~-400 mV.[8]

In pulse radiolysis studies with negatively charged $CO_2^{\bullet-}$ (-2.0 V)[9] as reductant the electrostatics are less favorable for reaction at the remote site. We have therefore explored also reaction with the positively charged Cd^+ as reductant (estimated reduction potential -1.80 V).[10] A preliminary account of the spinach Tyr83 studies has appeared.[11]

Experimental Section

Proteins. Procedures for isolation and purification of plastocyanin from spinach (*Spinacea oleracea*)[12-14] and parsley (*Petroselinum sativum*) have been described.[15-17] Homogeneity of the purified proteins was

† Present address: Chemistry Department A, Building 207, The Technical University of Denmark, 2800 Lyngby, Denmark.

‡ The Protein Laboratory, University of Copenhagen, Copenhagen, Denmark.

§ Cookridge Radiation Centre, University of Leeds, Cookridge Hospital, Leeds LS16 6QB, England.

(1) Sykes, A. G. *Chem. Soc. Rev.* **1985**, *14*, 285-315; *Struct. Bonding* **1991**, *75*, 175-224.
(2) Therien, M. J.; Selman, M.; Gray, H. B.; Chang, I.-J.; Winkler, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 2420 and references therein.
(3) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160.
(4) Liang, N.; Mauk, A. G.; Pielak, G. J.; Johnson, J. A.; Smith, M.; Hoffman, B. M. *Science* **1988**, *240*, 311.
(5) Durham, B.; Pan, L. P.; Long, J. E.; Millet, F. *Biochemistry* **1989**, *28*, 8659.
(6) Whitburn, K. D.; Hoffman, M. Z.; Simic, M. G.; Brezniak, N. V. *Inorg. Chem.* **1980**, *19*, 3180. Hoffman, M. Z.; Simic, M. G. *J. Am. Chem. Soc.* **1972**, *94*, 1757. Whitburn, K. D.; Hoffman, M. Z.; Brezniak, N. V.; Simic, M. G. *Inorg. Chem.* **1986**, *25*, 3037.
(7) Beitz, J. V.; Miller, J. R.; Cohen, H.; Wieghardt, K.; Meyerstein, D. *Inorg. Chem.* **1980**, *19*, 966.
(8) Neta, P.; Simic, M. G.; Hoffman, M. Z. *J. Phys. Chem.* **1976**, *80*, 2018.
(9) Breitenkemp, M.; Henglein, A.; Lilie, J. *Ber. Bunsen-Ges. Phys. Chem.* **1976**, *80*, 973; **1977**, *81*, 556.
(10) Meyerstein, D.; Navon, G. *J. Phys. Chem.* **1970**, *74*, 4067.
(11) Govindaraju, K.; Salmon, G. A.; Tomkinson, N. P.; Sykes, A. G. *J. Chem. Soc., Chem. Commun.* **1990**, 1003.
(12) Ellefson, W. L.; Ulrich, E. A.; Krogmann, D. W. *Methods Enzymol.* **1980**, *69*, 223.
(13) Gross, E. L.; Anderson, G. P.; Ketchner, S. L.; Draheim, J. E. *Biochim. Biophys. Acta* **1985**, *808*, 437.
(14) Davis, D. J.; San Pietro, A. *Arch. Biochem. Biophys.* **1977**, *182*, 266.
(15) Plesnicar, M.; Bendall, D. S. *Biochim. Biophys. Acta* **1970**, *216*, 192.
(16) Wood, P. M. *Biochim. Biophys. Acta* **1974**, *357*, 370.

0020-1669/93/1332-0040$04.00/0

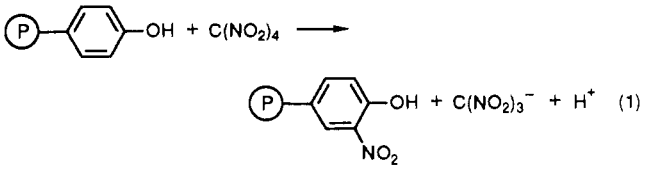
Table I. Products from the Reaction of Parsley Plastocyanin with Tetranitromethane[a]

FPLC fractions	% yield[b]	NO_2/Cu[c]	FPLC fractions	% yield[b]	NO_2/Cu[c]
band 1	28	native	band 4	8.5	~2.0
band 2	14	1.03	band 5	5.5	>2.0
band 3	32	~1.0	band 6		denatured

[a] $[PCu^{II}] = 0.32$ mM, $[C(NO_2)_4] = 10$ mM, pH = 8.0 (50 mM Tris/HCl + 0.1 M NaCl). Reaction time = 50 min. [b] Purified product as % yield of initial amount. [c] From UV–vis spectra.

confirmed by chromatography on a Mono-Q FPLC (Pharmacia) column in 20 mM Tris buffer at pH 7.50. Proteins in the Cu(II) state with absorbance (A) ratios $A_{278}/A_{597} < 1.14$ (spinach) and $A_{278}/A_{597} = 1.68$ (parsley) were used for modification. The different ratios are due to different aromatic compositions.

NO_2 Modification of Plastocyanin. The modification of spinach plastocyanin, PCu^{II}, was carried out according to published procedures (eq 1).[18-20] The procedure in the case of parsley plastocyanin was as



(1)

follows. To a stirred solution of PCu^{II} (0.32 mM) in Tris buffer (50 mM, pH 8.0) with NaCl (100 mM) was added 30 μL of tetranitromethane (Sigma Chemical Co. Ltd.; 0.84 M in absolute ethanol) every 10 min for a total of 30 min at 20 °C. The reaction was terminated after a total reaction time of 50 min by passing the reaction mixture through a Sephadex G25-150 (3.5 × 15 cm) gel column equilibrated with Bis-Tris buffer (20 mM, pH 6.0).

Purification of Products. The NO_2-modified plastocyanins were separated and purified by Pharmacia FPLC. A Mono-Q anion-exchange column (HR 5/5) equilibrated with Tris buffer (20 mM, pH 7.5) was used. Proteins were eluted with a linear NaCl gradient consisting of (A) Tris buffer (20 mM, pH 7.5), and (B) Tris buffer (20 mM, pH 7.5) with 1 M NaCl. A gradient of 1.5% NaCl/mL with a flow rate of 1.5 mL/min was used. The extent of modification was determined by measuring the concentration of plastocyanin at 597 nm ($\epsilon = 4500\ M^{-1}\ cm^{-1}$)[21] and the NO_2 peak at 355 nm. There is a pH-dependent isosbestic point for NO_2-modified tyrosine at 381 nm ($\epsilon = 2200\ M^{-1}\ cm^{-1}$).[22] The singly-modified plastocyanins were collected and stored in Bis-Tris buffer (60 mM, pH 6.0). Only one singly-modified derivative was obtained in the case of spinach plastocyanin,[18-20] whereas two (bands 2 and 3) were obtained in the case of parsley plastocyanin (Table I). These were obtained alongside bands assigned as unmodified native protein (band 1), multiply modified fractions (bands 4 and 5), and denatured protein (band 6), Figure 1.[23] Further purification of bands 2 and 3 was achieved by repeated FPLC separation using a smaller linear gradient (1.0% NaCl/mL) with reduced flow rate (1.0 mL/min).

UV–Vis Spectra. The singly-modified products of spinach and parsley plastocyanin have similar spectra, with a new peak at 355 nm, ϵ 3900 $M^{-1}\ cm^{-1}$, at pH 6.0 assigned to the $TyrNO_2$ component, Figure 2. On ascorbate reduction, $PCu^{II} \rightarrow PCu^{I}$, the 597-nm peak disappears with an increase in absorption at 278 nm.[13,19] The $TyrNO_2$ peak at 355 nm is pH dependent, pK_a 8.10 for the Cu(II) state, and shifts toward longer wavelengths (428 nm) as the pH is increased, due to phenolic acid/phenolate interconversion.[18]

Peptide Mapping. A procedure for peptide mapping of the NO_2 derivative of spinach plastocyanin has already been reported.[20] Confirmation of parsley bands 2 and 3 in Figure 1 as single modified products was carried out with some slight modification of the published procedure.[20]

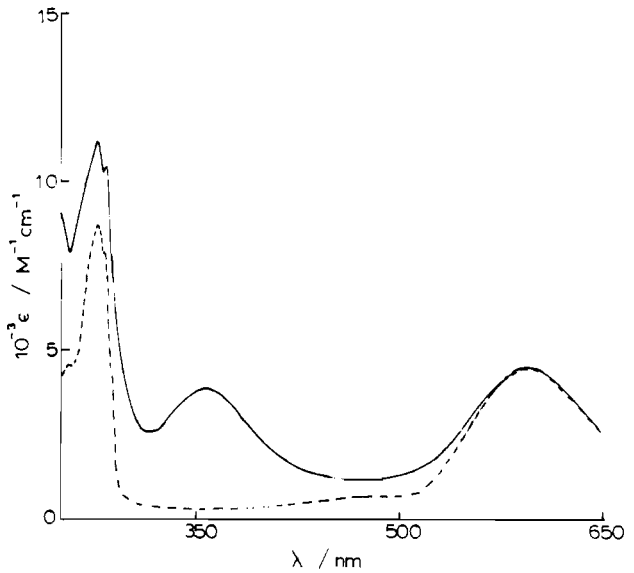


Figure 2. Comparison of UV–vis spectra of parsley PCu^{II} plastocyanin native (- - -) and Tyr62 (or Tyr83) NO_2-modified at pH 6.0 (—).

Thus 0.5-mg samples of FPLC-purified NO_2-modified bands 2 and 3 and native PCu^{II}, in 100 mM NH_4HCO_3, were heat-denatured separately and digested for 3 h using TPCK-treated trypsin type XIII (Sigma; 1 mg in 10 mL of 1.00 M HCl). The digestions were terminated by freezing samples. The peptides were in each case lyophilized using a HetoSicc freeze-dryer. The tryptic peptides were separated on a Waters HPLC equipped with a photodiode array detector using a delta-pak C^{18} 300-Å (3.9 mm × 15 cm) reverse-phase column. The peptides were eluted with 0–60% linear gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in a 9:1 acetonitrile/water mixture over 24 min with flow rate of 1.3 mL/min, Figure 3 (see also Figure 4[23]). The tryptic peptides were partly identified by UV–vis spectra, Figures 5 (see also Figure 6[23]) of the $TyrNO_2$-containing peptides and were further confirmed by sequence analysis using a gas-phase microsequencer (Applied Systems Model 470A), equipped with an on-line HPLC (Applied Systems Model 120A) for analysis of the released phenylthiohydantoin amino acids.

The peptides were separated into eight main and a number of smaller fractions. The main fractions containing $TyrNO_2$ were identified as peptide seven (P7) in band 2 (peak e in Figures 3 and 5) and peptide five (P5) in band 3 (peak f in Figures 4 and 6),[23] numbered in accordance with the expected cleavage points in the amino acid sequence (Figure 7). These two fractions (P7 in band 2 and P5 in band 3) have counterpart peaks in the chromatogram of native parsley plastocyanin, where retention times are 1.68 and 0.76 min shorter than those for the NO_2-modified proteins.

Amino acid sequencing of band 2 has confirmed the $TyrNO_2$-containing fragment to be P7, which contains Tyr83 as the only tyrosine. The NO_2-tyrosine fragment obtained from the HPLC separation of band 3 after trypsin digestion was contaminated with other peptides. Thus, on further HPLC separation this gave three different fractions. Fraction 1 turned out to be an N-terminal-blocked fraction, and no sequence could be determined. Fraction 2 was found to be the P2 peptide, with a small amount of P4 peptide as impurity. The third fraction was confirmed to be the P5 peptide and contained a $TyrNO_2$ moiety (absorbance at ~355 nm). The sequence of P5 was confirmed up to 18 amino acid from a total of 21, and the position of NO_2 modification shown to be on Tyr62. Figure 8 illustrates the positions of Tyr83 and Tyr62 relative to the imidazole ring of His87, which is coordinated to the Cu.

Pulse Radiolysis. All solutions were prepared in distilled water further purified by milli Q ion-exchange, with 40 mM (for $CO_2^{\bullet-}$) or 0.1 mM (for Cd^+) phosphate buffer. Experiments were carried out at the Cookridge Research Centre, using a 1-cm light path cell and a beam of 2.5-MeV electrons. Pulse lengths were either 0.2 or 0.6 μs. The yields of reducing radical (R) for a given pulse were calculated from (2). The

$$[R] = V(SEC) \times S(SEC) \times G(R) \qquad (2)$$

secondary emission-chamber voltage, V(SEC), was measured directly,

(17) Segal, M. G.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4585.
(18) Davis, D. J.; San Pietro, A. *Anal. Biochem.* **1979**, *95*, 254.
(19) Anderson, G. P.; Draheim, J. E.; Gross, E. L. *Biochim. Biophys. Acta* **1985**, *810*, 123.
(20) Christensen, H. E. M.; Ulstrup, J.; Sykes, A. G. *Biochim. Biophys. Acta* **1990**, *1039*, 94.
(21) Ramshaw, J. A. M.; Brown, R. H.; Scanten, M. D.; Boulter, D. *Biochem. Biophys. Acta* **1973**, *303*, 269.
(22) Riorden, J. F.; Sokolovsky, M.; Vallee, B. L. *Biochemistry* **1967**, *6*, 358.
(23) See paragraph at end of paper regarding supplementary material.

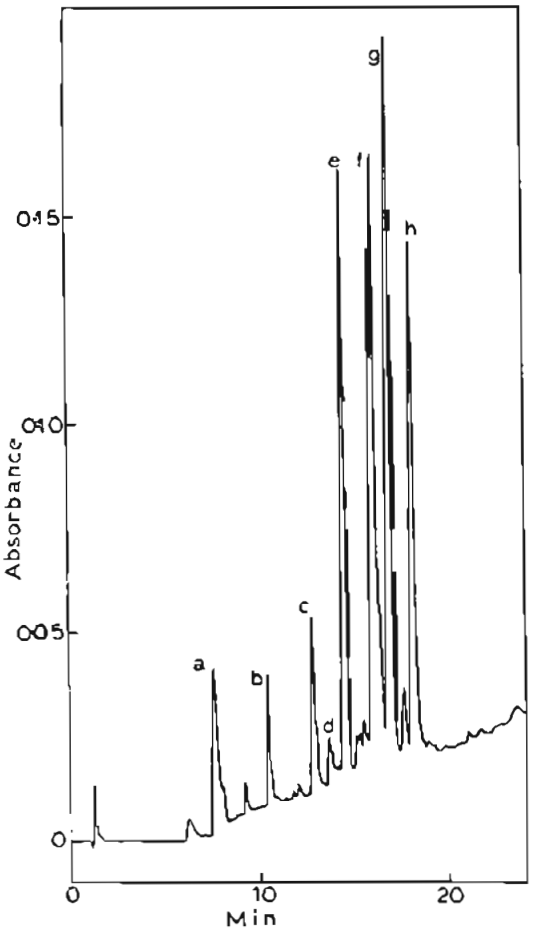


Figure 3. HPLC separation of trypsin-digested products from band 2 of parsley $PCu^{II}Tyr83NO_2$ (Figure 1), monitored at 220 nm.

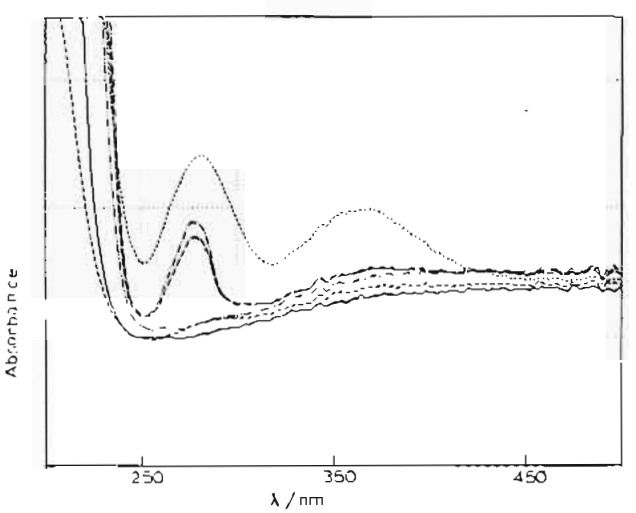


Figure 5. UV–vis spectra of tryptic peptides from the chromatogram for $PCu^{II}Tyr83NO_2$ in Figure 3 (same lettering), with peaks for c (—), d (- - -), e (···), two fractions of f [initial (- -) and middle (- ·-)], and g (- ·· -).

and the sensitivity, S(SEC), obtained by thiocyanate dosimetry.[24] Here G(R) is the radiation chemical yield of radicals per J of energy absorbed by the system.

Pulse radiolysis experiments were at 20 ± 1.5 °C, with N_2O- or Ar-saturated solutions containing oxidized protein PCu^{II} (spinach and parsley), $PCu^{II}Tyr83NO_2$ (spinach and parsley), or $PCu^{II}Tyr62NO_2$ (parsley). The ionic strength was adjusted to I = 0.100 M with NaO_2CH or Na_2SO_4.

(24) Fielden, E. M. In *The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis*; Baxendale, J. H., Busi, F., Eds.; Riedel: New York, 1982; p 58

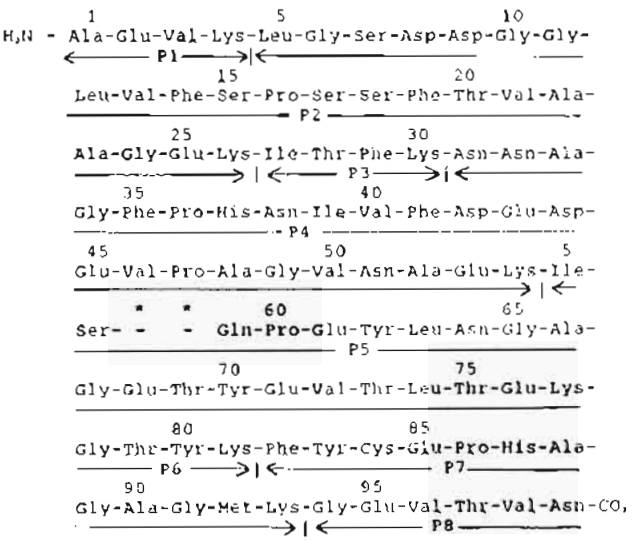


Figure 7. Primary structure of parsley plastocyanin. The peptides produced by trypsin cleavage of native and NO_2-modified protein are the same. Two deletions at 57 and 58 are indicated by asterisks.

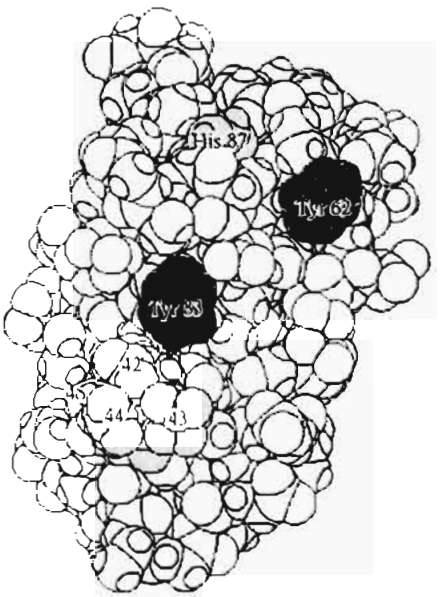


Figure 8. Space-filling molecular graphics representations of parsley plastocyanin based on the *Enteromorpha prolifera* X-ray crystal structure,[28] illustrating the relative positions of Tyr83 (and the adjacent 42–45 acidic patch), Tyr62, and active site His87.

Experiments were also carried out on samples of reduced proteins, $PCu^{I}Tyr83NO_2$ (spinach and parsley) and $PCu^{I}Tyr62NO_2$ (parsley). Protein concentrations were in the range 9–65 μM. Samples of NO_2-modified proteins were purified by FPLC, the day prior to kinetic experiments. They were then oxidized (with $[Fe(CN)_6]^{3-}$), or reduced (with ascorbate), and exhaustively diafiltered into 40 or 0.1 mM phosphate buffer generally at pH 7.0.

Solutions also contained sodium formate (0.012 M) or cadmium sulfate (100 μM) to generate $CO_2^{\bullet-}$ or Cd^{+} radicals, respectively, on pulsing. In experiments using $CO_2^{\bullet-}$ as reductant, the protein samples were saturated with N_2O gas, whereas, in the case of Cd^{+} reduction, the samples were saturated with Ar gas and contained 10^{-2} M *tert*-butyl alcohol to scavenge OH radicals. Whereas $CO_2^{\bullet-}$ is the sole radical product in formate solutions, in the Cd^{2+}/*tert*-butyl alcohol system, along with Cd^{+}, unreactive *tert*-butyl alcohol radicals are also formed. Although reduction potentials are high, for $CO_2^{\bullet-}$ (−2.0 V)[9] and Cd^{+} (−1.8 V),[10] these are lower than that of e_{aq}^{-} (−2.9 V)[25] and are less likely therefore to undergo reaction at sites other than NO_2 and Cu(II). An estimate of the efficiency of the reduction was made by comparing the yields of $CO_2^{\bullet-}$ or Cd^{+} with the

(25) Swallow, A. J. *Radiation Chemistry*; Longmans: New York, 1973; p 148.

amount of protein reduced. With knowledge of these efficiencies the dose was adjusted so that expected reduction of $PCu^{II}TyrNO_2$ or $PCu^{I}TyrNO_2$ was <10%.

In a typical experiment with 22 μM of protein the dose was 4.2 G_γ. Absorbance (A) changes were calculated using (3), where V is the transient signal from the photomultiplier and V_0 the signal before the radiation pulse.

$$A = -\log (1 - V_s/V_0) \quad (3)$$

Concentrations of Cu(II) were monitored at 597 nm, while $TyrNO_2^{\bullet-}$ and Cd^+ both absorb at 300 nm.[6] There is a small absorbance change due to the $PCu^{II} \rightarrow PCu^{I}$ change at 300 nm. Filters were placed before the sample cell to prevent the transmission of second-order spectra by the monochromator and to exclude unwanted light which may induce photochemical reactions. Light of wavelengths below 550 nm was excluded by an OG550 filter for experiments at 597 nm. A UG5 filter was used for experiments at 300 nm to exclude wavelengths below 230 nm. Typical traces for the reduction of Cu(II) at 597 nm, and for Cd^+ formation and decay (first pulse), are shown in Figure 9a,b. The residual absorption at the end of the trace in Figure 9b is attributed to absorption by PCu^{I}. Subsequent pulses show an increase in residual absorption at the end of the trace which is due to the formation of $TyrNO_2^{\bullet-}$ (Figure 9c) with correspondingly less reduction at Cu(II). In Figure 9d a trace at 300 nm for the reduction of $PCu^{I}Tyr83NO_2$ by Cd^+ is shown. The ratio of the peak absorption to that at the plateau in the time profile reflects the relative absorption coefficients of Cd^+ and $PCu^{I}Tyr83NO_2^{\bullet-}$.

Treatment of Data. Pulse radiolysis traces retrieved from disk files were subjected to first-order kinetic analysis using a computer program TREAT written by Dr. F. Wilkinson.[26] Plots of $\ln\{(A_\infty - A_t)/(A_\infty - A_0)\}$ against time were linear to at least 3 half-lives, and first-order rate constants k_{obs} were obtained from these by a standard linear least-squares procedure.

Results

Spinach Plastocyanin. The reduction of native PCu^{II} (17–49 μM) with $CO_2^{\bullet-}$ monitored at 597 nm gives only one stage with a rate constant of $(3.0 \pm 0.5) \times 10^8$ M^{-1} s^{-1}. In the case of the NO_2 derivative, $PCu^{II}Tyr83NO_2$, absorbance changes were monitored at the Cu(II) peak at 597 nm. A fast reduction was observed, rate constants $(5.7 \pm 0.01) \times 10^8$ M^{-1} s^{-1}, whereas at 300 nm no formation of $TyrNO_2^{\bullet-}$ ($\epsilon \sim 10^4$ M^{-1} cm^{-1})[6,8] was observed after the first pulse. Runs at different protein concentrations were carried out. Second-order rate constants, Table II, were obtained from the slopes in Figure 10. In the case of reduction by $CO_2^{\bullet-}$ the positive intercepts are due to the bimolecular decay of the radicals.

At this stage it was decided that $CO_2^{\bullet-}$ may not be efficient in reducing $Tyr83NO_2$ due to the effect of negatively charged residues (42–45 and 59–61) at this site. Hence, the positively charged reductant Cd^+ was used. However, similar results were obtained with Cd^+, which like $CO_2^{\bullet-}$ is generated rapidly, in this case by reaction of e_{aq}^- with 100 μM Cd^{2+}. The rate constant for Cu(II) $\rightarrow$ Cu(I) reduction of the $Tyr83NO_2$ protein by Cd^+ $(4.9 \pm 0.5) \times 10^9$ M^{-1} s^{-1} compares with $(1.53 \pm 0.40) \times 10^9$ M^{-1} s^{-1} for native protein. Two experiments with e_{aq}^- as reductant gave a rate constant for the NO_2-modified derivative at $<1.2 \times 10^9$ M^{-1} s^{-1}, which is less than the rate constant for Cd^+. With Cd^+ the more pronounced intercepts are due to the corresponding decay of Cd^+ and the reaction of Cd^+ with the mildly oxidizing *tert*-butyl alcohol radical.

In experiments on the reduced protein, $PCu^{I}Tyr83NO_2$, formation of $TyrNO_2^{\bullet-}$ was observed, rate constant $(4.2 \pm 0.5) \times 10^9$ M^{-1} s^{-1}, as the main process, on the first pulse, Figure 9d. This is followed by slow decay, believed to correspond to electron transfer from $TyrNO_2^{\bullet-}$ to some unidentified site/species. For solutions with 13.2 μM $PCu^{I}Tyr83NO_2$ the decay process following the first pulse occurs over 800 ms. For completeness we note that this unidentified site appears to be saturated after 3–4 pulses, i.e. ~30% reduction. Also for solutions containing both the Cu(II) and Cu(I) protein when saturation is complete, a slow intermolecular reduction of Cu(II) occurs ($\sim 1 \times 10^5$ M^{-1} s^{-1}). These processes are not considered important alongside the previously mentioned reactions.

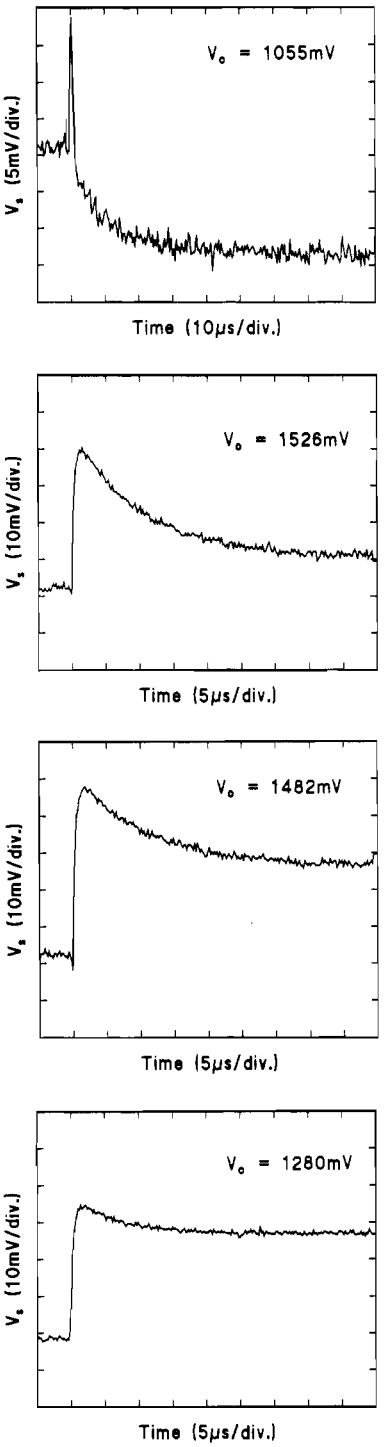


Figure 9. Pulse radiolysis traces for the reaction of Cd^+ with (top to bottom) (a) $PCu^{II}Tyr83NO_2$ observed at 597 nm (first pulse), (b) $PCu^{II}Tyr83NO_2$ at 300 nm (first pulse), (c) $PCu^{II}Tyr83NO_2$ at 300 nm (ninth pulse), and (d) $PCu^{I}Tyr83NO_2$ at 300 nm (first pulse).

Parsley Plastocyanin. The reaction of native PCu^{II} (17–65 μM) with Cd^+ gives only one stage with rate constant $(1.0 \pm 0.13) \times 10^9$ M^{-1} s^{-1} from absorbance changes at 597 nm. Reduction of both the $PCu^{II}Tyr83NO_2$ and $PCu^{II}Tyr62NO_2$ derivatives with Cd^+ give similar behavior. Thus, rate constants for the reduction of Cu(II) (at 597 nm) are $(5.5 \pm 0.1) \times 10^9$ and $(5.2 \pm 0.2) \times 10^9$ M^{-1} s^{-1} respectively, Table II.

(26) Cookridge Radiation Research Centre, internal report, Feb 1988.

Table II. Summary of Rate Constants (~20 °C) at pH 7.0 (Phosphate) for the First Stage of the Reduction of Spinach and Parsley Plastocyanins and Their Tyr83 and Tyr62 NO_2-Modified Derivatives with I = 0.10 M (NaO_2CH or Na_2SO_4)

reacn	$k_1/M^{-1}\ s^{-1}$
Spinach	
$PCu^{II} + CO_2^{\bullet-}$	3.0 (0.5) × 10^8
$PCu^{II}Tyr83NO_2 + CO_2^{\bullet-}$	5.7 (0.1) × 10^8
$PCu^{II} + Cd^+$	1.5 (0.4) × 10^9
$PCu^{II}Tyr83NO_2 + Cd^+$	4.9 (0.5) × 10^9
$PCu^{I}Tyr83NO_2 + Cd^+$	4.2 (0.5) × 10^9
Parsley	
$PCu^{II} + Cd^+$	1.0 (0.1) × 10^9
$PCu^{II}Tyr83NO_2 + Cd^+$	5.5 (0.1) × 10^9
$PCu^{I}Tyr83NO_2 + Cd^+$	3.0 (0.3) × 10^9
$PCu^{II}Tyr62NO_2 + Cd^+$	5.2 (0.2) × 10^9
$PCu^{I}Tyr62NO_2 + Cd^+$	2.8 (0.1) × 10^9
A. variabilis	
$PCu^{II} + Cd^+$ [a]	3.0 (0.5) × 10^8

[a] PCu^{II} = 15–67 μM. For the growing and isolation procedures, see: Jackman, M. P.; Sinclair-Day, J. D.; Sisley, M. J.; Sykes, A. G. *J. Am. Chem. Soc.* **1987**, *109*, 6443.

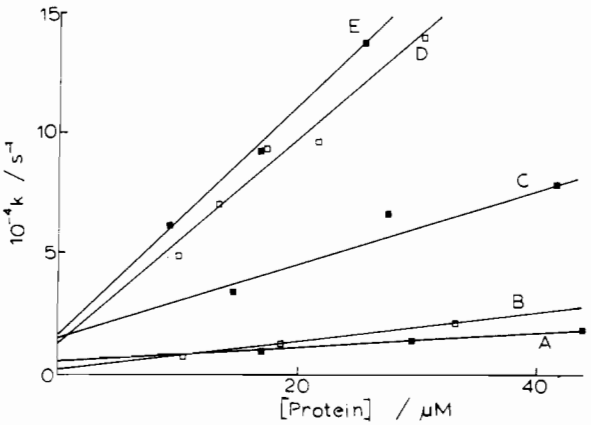


Figure 10. Dependence of first-order rate constants, k_{obs} (20 °C), on protein concentration for the reduction of spinach plastocyanin (and derivatives) at pH 7.0 (phosphate), I = 0.10 M (NaO_2CH or Na_2SO_4): (A) $PCu^{II} + CO_2^{\bullet-}$; (B) $PCu^{II}Tyr83NO_2 + CO_2^{\bullet-}$; (C) $PCu^{II} + Cd^+$; (D) $PCu^{I}Tyr83NO_2 + Cd^+$; (E) $PCu^{II}Tyr83NO_2 + Cd^+$.

As in the case of the spinach derivative, formation of $TyrNO_2^{\bullet-}$ is not observed during the first pulse on parsley $PCu^{II}Tyr83NO_2$ and $PCu^{II}Tyr62NO_2$. Subsequently, on further pulsing, as the PCu(I) product build up, increasing yields of $TyrNO_2^{\bullet-}$ are obtained. Again the transient $TyrNO_2^{\bullet-}$ undergoes slow decay, transferring electrons to an unidentified site/species. After saturation of this site intermolecular reduction of Cu(II) on another molecule is observed with rate constant 5.4 × 10^5 M^{-1} s^{-1} for $PCu^{II}Tyr83NO_2$ and 2.4 × 10^5 M^{-1} s^{-1} for $PCu^{II}Tyr62NO_2$.

In the case of $PCu^{I}Tyr83NO_2$ and $PCu^{I}Tyr62NO_2$, formation of the $TyrNO_2^{\bullet-}$ radical is observed from the first pulse, rate constants (3.0 ± 0.3) × 10^9 M^{-1} s^{-1} and (2.8 ± 0.05) × 10^9 M^{-1} s^{-1}, respectively. The slow decay of $TyrNO_2^{\bullet-}$ is also observed. However in sharp contrast to the Cu(II) studies, the $TyrNO_2^{\bullet-}$ radical (spinach as well as parsley) is stable for a longer time after saturating the unknown site or species.

The first and second stages of the reaction of spinach $PCu^{II}Tyr83NO_2$ and parsley $PCu^{II}Tyr83NO_2$ and $PCu^{II}Tyr62NO_2$ are summarized in (4). The various rate

$$CO_2^{\bullet-}/Cd^+ + PCu^{II}TyrNO_2 \xrightarrow{k_1} \begin{cases} PCu^{I}TyrNO_2 \\ + CO_2/Cd^{2+} \end{cases} \qquad PCu^{II}TyrNO_2^{\bullet-} \xrightarrow{k_2} PCu^{I}TyrNO_2 \qquad (4)$$

constants are listed in Table II. In all cases k_2 is fast and estimated at $>10^7$ s^{-1}. In these studies we are not able to determine a more precise k_2 because $PCu^{II}TyrNO_2^{\bullet-}$ cannot be generated any faster.

Discussion

Only one out of three tyrosines (Tyr83) is NO_2-modified in the case of spinach plastocyanin. With parsley plastocyanin two products are obtained, and two out of four tyrosines are modified. From UV–vis spectra and the absorbances at 355 nm, ϵ = 3900 M^{-1} cm^{-1} for $TyrNO_2$ at pH <7.5, and 597 nm, ϵ = 4500 M^{-1} cm^{-1} for Cu(II), all three products are 1:1 modified derivatives. The peak at 597 nm remains unaltered indicating no perturbation of the PCu^{II} active site as a result of NO_2 modification. Consistent with this the reduction potential of spinach plastocyanin changes by only 20 mV on NO_2 modification.[20] From known organic reactivities the NO_2 group can be assumed to be ortho to the phenolic OH group. Enhanced acid dissociation of the phenolic group is observed. From peptide mapping, the two parsley products are modified by Tyr83 and Tyr62, respectively. Residues 70 and 80, which are not exposed at the surface, remain unmodified in the case of both plastocyanins.

The yields of 14% (Tyr83) and 32% (Tyr62), respectively, for parsley plastocyanin compare with 26% (Tyr83) for the spinach modification and indicate that Tyr62 is the more readily modified. The position of the Tyr83 residue at the surface of the protein as a component of the remote acidic patch region is well documented from crystallographic studies on both the poplar[27] and *Enteromorpha prolifera*[28] plastocyanins. The position remains unchanged for both the Cu(II) and Cu(I) proteins. It is believed that Tyr83 has some functional role in ET from the remote site.[29] Of the 25 plastocyanin sequences available, Tyr83 is present in all but *Scenedesmus obliquus* and *Chlorella fusca*,[30,31] in which case the highly conserved Phe82 and Tyr83 residues are interchanged, apparently with little effect on reactivity.[32] An aromatic residue either Tyr or Phe would seem to be a requirement therefore at position 83. Eight of the sequences so far determined have deletions at positions 57 and 58.[33] These are *C. fusca*, *S. obliquus*, *E. prolifera*, and *U. arasakii*, i.e., all the green algal plastocyanins so far sequenced as well as in parsley, barley, rice, and carrot plastocyanins.[34] Of particular interest is the tyrosine at position 62, which is present in all these but no other sequences.[34] Studies on both the X-ray crystal structure of *E. prolifera*[28] and *S. obliquus* from 2D NMR[35] have identified structural changes resulting from the two deletions. Thus, a kink in the peptide chain, which is a feature of the poplar structure, is not present in plastocyanins with deletions at 57 and 58, Figure 11. There is moreover less negative charge at residues 59–61. The position of the α-carbon of residue 62 does not appear to change with or without the deletions. It is however likely that Tyr62 compensates in some way for the loss of negative charge at 59–61 and like Tyr83 is functionally important in ET processes.

The pulse-radiolysis studies on NO_2-modified species define a new approach to studying intramolecular ET reactions of

(27) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521.
(28) Collyer, C. A.; Guss, J. M.; Sugimura, Y.; Yoshizaki, F.; Freeman, H. C. *J. Mol. Biol.* **1990**, *211*, 617.
(29) Brunschwig, B. S.; DeLaive, P. J.; English, A. M.; Goldberg, M.; Gray, H. B.; Mayo, S. L.; Sutin, N. *Inorg. Chem.* **1985**, *24*, 3743.
(30) Ambler, R. P. Unpublished work quoted in ref 1.
(31) Kelly, J.; Ambler, R. P. *Biochem. J.* **1974**, *143*, 681. *Plant Biochemistry* (II); Northcote, D. H., Ed.; University Park: Baltimore, MD, 1977; Vol. 13, pp 1–40.
(32) McGinnis, J.; Sinclair-Day, J. D.; Sykes, A. G.; Powls, R.; Moore, J.; Wright, P. E. *Inorg. Chem.* **1988**, *27*, 2306.
(33) Sykes, A. G. *Adv. Inorg. Chem.* **1991**, *36*, 377–408.
(34) For recent sequences, see e.g.: Yano, H.; Kamo, M.; Tsugita, A.; Aso, K.; Nozu, Y. *Protein Sequence Data Anal.* **1989**, *2*, 385. Last, D. I.; Gray, J. C. *Plant Mol. Biol.* **1989**, *12*, 655. Yoshizaki, F.; Fukazawa, T.; Mishina, Y.; Sugimura, Y. *J. Biochem.* **1989**, *106*, 282.
(35) (a) Moore, J. M.; Case, D. A.; Chazin, W. J.; Gippert, G. P.; Havel, T. F.; Powls, R.; Wright, P. E. *Science* **1988**, *240*, 314. (b) Moore, J. M.; Chazin, W. J.; Powls, R.; Wright, P. E. *Biochemistry* **1988**, *27*, 7806.

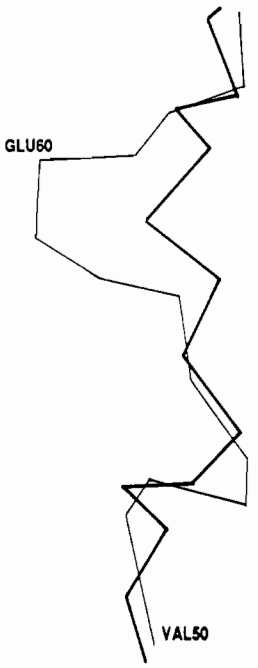


Figure 11. Superposition of polypeptide backbones for spinach (no deletions) and parsley plastocyanin (deletions at 57 and 58) in the region 50–62, based on information from X-ray crystal and 2D NMR studies.[28,35]

metalloproteins. In the present studies each pulse had a reducing capacity of <10% of the total protein (9–65 μM). The pH was adjusted to 7.0 with 40 mM phosphate for $CO_2^{\bullet-}$ and 0.10 mM phosphate for Cd^+ experiments (to avoid precipitation), $I = 0.100$ M (NaO_2CH or Na_2SO_4). In experiments using $PCu^{II}Tyr83NO_2$, with sodium formate (12 mM) to generate $CO_2^{\bullet-}$, the first pulse gave no evidence for $NO_2^{\bullet-}$ formation at 300 nm ($\epsilon \sim 10^4$ M^{-1} cm^{-1}). The Cu(II) → Cu(I) reduction monitored at 597 nm is uniphasic giving a rate constant of 5.7×10^8 M^{-1} s^{-1} (spinach). The smaller rate constant obtained on pulse radiolysis of the corresponding native Cu(II) plastocyanin (3.0×10^8 M^{-1} s^{-1}) is noted. Subsequent pulsing of the NO_2-modified derivative gives increasing amounts of $NO_2^{\bullet-}$ with correspondingly less Cu(II) reduction. This process is assigned to the reduction of NO_2 of $PCu^{I}TyrNO_2$, which is a product from the previous pulsing. Bimolecular decay of $PCu^{I}TyrNO_2^{\bullet-}$ by reaction with PCu^{II}. $TyrNO_2$ is observed as a slower stage to reaction.

One possible consideration in the case of both $PCu^{II}Tyr83NO_2$ derivatives is that $CO_2^{\bullet-}$ may not be a very effective reductant at the Tyr83 site because of the substantial negative charge in this region. In further experiments positively charged Cd^+ was used therefore as reductant. Again there was no observable buildup of $NO_2^{\bullet-}$ in the first pulse, and uniphasic kinetics were observed. Rate constants for Cd^+ were 1 order of magnitude greater than those for $CO_2^{\bullet-}$ consistent with an effect of charge. In this context we note also that the rate constant for reaction of Cd^+ with the basic *Anabaena variabilis* PCu^{II} plastocyanin (+2 charge) is 1 order of magnitude less than rate constants for spinach (–8) and parsley (–7) PCu^{II}, Table II. However, particularly important in Table II is the observation that all rate constants for NO_2-modified derivatives are greater than those for native protein.

In experiments on reduced protein, $PCu^{I}Tyr83NO_2$ or $PCu^{I}Tyr62NO_2$, formation of $NO_2^{\bullet-}$ was observed as the prime process. The 2–5-fold greater rate constant for $CO_2^{\bullet-}$ and Cd^+ reduction of Cu(II) of $PCu^{II}TyrNO_2$ as compared to native protein indicates a significant supplement to ET from reduction of Cu(II) via the NO_2 group. The through-space distances from Cu to C^γ of the tyrosines are quite long at 9.2 Å (Tyr83) and 8.6 Å (Tyr62).[27,28] There is no structural or spectroscopic evidence for changes at the tyrosines with change in oxidation state at the Cu. It can be assumed therefore that $TyrNO_2^{\bullet-}$ is generated at about the same rate in the Cu(II) case. Because no $Tyr^{\bullet-}$ absorption is observed at 300 nm, rapid $>10^7$ s^{-1} intramolecular ET $PCu^{II}Tyr^{\bullet-} \rightarrow PCu^{I}TyrNO_2$ must follow. An opposing view that no $TyrNO_2^{\bullet-}$ is generated seems extremely unlikely (there is no obvious way in which the NO_2 might be protected).

In the case of the $PCu^{I}TyrNO_2$ derivatives, the $NO_2^{\bullet-}$ radical which is formed decays by a slow intra- or intermolecular process to another site or species, which has not yet been identified. For a solution of 13.2 μM spinach $PCu^{I}Tyr83NO_2$ for example the decay following the first pulse occurs over 800 ms, but the site or species to which ET occurs is saturated after 3–4 pulses, i.e. after ~30% reduction. We regard this process to be of minor importance in the context of present studies. In experiments on solutions containing the Cu(I) and Cu(II) states, when this saturation is complete, a slow intermolecular reduction of Cu(II) is observed.

The fast intramolecular rate constant $>10^7$ s^{-1} in both the $PCu^{II}Tyr83$ and $PCu^{II}Tyr62$ studies is of interest in the context of ET pathways in metalloproteins. The direct edge to edge distances based on the crystal structure of poplar plastocyanin are 7.7 Å for Tyr83 (nearest point on the aromatic ring to the cysteinyl sulfur) and 6.9 Å for Tyr62 (C^γ of Leu62) to the imidazole of His37.[27] A recent advance in this area has been made by Beratan and colleagues following the development of a computer program which is capable of searching for pathways through proteins based on the crystal-structure coordinates.[36–39]

In this approach the ET rate constant for weakly coupled donor–acceptor sites in proteins can be expressed as (5), where T_{DA} is

$$k_{et} = (2\pi/h)(T_{DA})^2(FC) \qquad (5)$$

the tunneling matrix element and is a measure of the electronic coupling. The Franck–Condon factor FC can be expressed as in eq 6, where ΔG is the driving force for the reaction and λ the

$$FC = (4\pi\lambda kT)^{-1/2} \exp[-(\lambda + \Delta G°)^2/4\lambda kT] \qquad (6)$$

reorganization energy. Using the crystal structure, the tunneling matrix element is calculated from combinations of covalent, hydrogen-bond, and through-space interactions that together constitute a pathway according to (7), and the program searches

$$T_{DA} = \text{prefactor } \pi_i\epsilon_i \qquad (7)$$

the protein for combinations of interactions that maximize $\pi_i\epsilon_i$. The contribution to the decay of the coupling for covalent interactions is fixed, while the decay for hydrogen-bond and through-space interactions decreases exponentially with distance.

Using the crystal-structure coordinates for poplar plastocyanin, and a value for β (the intrinsic coupling capability of the protein) of 0.98 Å, the best ET pathways in the system have been systematically investigated. For the Tyr83 derivative, the pathway with the best electronic coupling involves seven covalent bonds from C^γ of Tyr83 to S of Cys84, Figure 12, $\pi_i\epsilon_i = 2.80 \times 10^{-2}$ (d = 10.8 Å). This pathway has a coupling value 10× that of the next best pathway identified by the program and is regarded favorably therefore. In order to identify pathways from Tyr62 to the active site, C^γ of Leu62 in the poplar structure was taken as the starting point for the transfer, since poplar plastocyanin has no tyrosine at this position. The best pathway was found to involve nine covalent bonds and one hydrogen bond from the C^γ at position 62 to the N^δ of His37, Figure 12, $\pi_i\epsilon_i = 4.03 \times 10^{-3}$ (d = 15.9 Å).

(36) Beratan, D. N.; Onuchic, J. N.; Betts, J. N.; Bowler, B. E.; Gray, H. B. *J. Am. Chem. Soc.* **1990**, *112*, 7915.
(37) Beratan, D. N.; Onuchic, J. N. *Photosynth. Res.* **1989**, *22*, 173.
(38) Beratan, D. N.; Onuchic, J. N.; Hopfield, J. J. *J. Chem. Phys.* **1987**, *86*, 4488.
(39) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285.

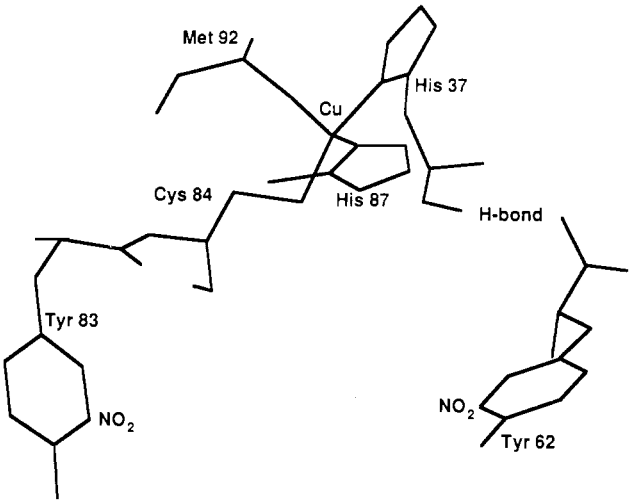


Figure 12. Pathways for electron transfer to Cu of plastocyanin from the Tyr83 and Tyr62 NO_2-modified sites, based on the poplar crystal structure,[2b] with Tyr62 replacing Leu62.

The pathways generated for the Tyr83 and Tyr62 derivatives are both favorable and have large electronic coupling values. On this basis the ET process would be expected to be very favorable, as indeed is the case. An estimate of the relative rate constants for the Tyr83 and Tyr62 derivatives can be obtained from a comparison of the values of $(T_{DA})^2$ for each derivative. Calculation of $[T_{DA}(\text{Tyr83Cys84})]^2/[T_{DA}(\text{Tyr62His37})]^2$ predicts that the Tyr83 rate should be ~48 times faster than the corresponding Tyr62 rate. Calculations have also been carried out to obtain estimates of rate constants based on the coupling values for Tyr83 and Tyr62. The calculations have been referenced to the well-characterized cytochrome *c* system.[40] Since the reorganization energy (λ) for the ET reaction of $NO_2^{\bullet-}$ to Cu(II) is not known accurately, calculations are here based on two estimates of λ, 0.30 eV (for the blue copper site alone) from studies on Ru-modified azurin,[41] and 1.21 eV from recent studies on intramolecular ET from the disulfide radical $RSSR^{\bullet-}$ to the Cu, again for azurin.[42] The value of λ in the latter case includes contributions from the Cu site, as well as disulfide and water molecules in close proximity.

Using eq 5, calculated rate constants can be obtained from (8), with $k_{ref} = 30\ s^{-1}$ ($-\Delta G° = 0.19$ eV). The $\Delta G°$ value for

$$\frac{k}{k_{ref}} = \frac{(T_{DA})^2(FC)}{(T_{DA_{ref}})^2(FC)_{ref}} \qquad (8)$$

intramolecular ET from $NO_2^{\bullet-}$ to Cu(II) used in this calculation is 0.8 eV. Calculations of this kind have been carried out previously for Ru-modified cytochrome b_5.[43] Here the rate constants calculated for the Tyr83 and Tyr62NO_2 derivatives vary significantly depending on the value of λ used. For the value 0.30 eV, they are $7.2 \times 10^5\ s^{-1}$ and $1.5 \times 10^4\ s^{-1}$, and for 1.21 eV, they are $2.9 \times 10^8\ s^{-1}$ and $6.0 \times 10^6\ s^{-1}$, respectively. The experimentally determined rate constants of $>10^7\ s^{-1}$ are in satisfactory agreement with the latter. The reactions of the radicals of the NO_2 derivatives are in some respects similar to that of $RSSR^{\bullet-}$ on azurin ($k = 44\ s^{-1}$).[42] The faster rate constant in the present case is attributed to the stronger coupling ($\pi_i\epsilon_i = 2.8 \times 10^{-2}$ for Tyr83 and 4.0×10^{-3} for Tyr62) and higher driving force ($-\Delta G° \sim 0.8$ eV).

The rapidity of ET from $NO_2^{\bullet-}$ to Cu(II) $>10^7\ s^{-1}$ contrasts sharply with the intramolecular rate constants reported for two His59 Ru-modified plastocyanins.[44] Rate constants for the Ru(II) → Cu(II) intramolecular ET are $<0.08\ s^{-1}$ and $<0.26\ s^{-1}$, respectively, over ~12 Å (driving force 250 mV). Although there are pathways from His59 to the active site that have only a single hydrogen bond and covalent connections,[45] these pathways appear to be unfavorable. The predicted coupling for His59 plastocyanin, $\pi_i\epsilon_i = 7.1 \times 10^{-5}$,[44] is significantly weaker than for the NO_2 derivatives examined in this study, with the driving force substantially lower and the distance greater, which could account for the differences between the two systems. Experiments at higher driving force would be extremely informative in this context and may help to resolve the issue.

To conclude, the procedure for NO_2 modification of tyrosine residues provides a new approach in the study of metalloprotein intramolecular ET reactions. Procedures for preparation and characterization of such derivatives have been described, and the pulse radiolysis methodology has been defined. We are at present investigating other proteins with tyrosines available for NO_2 modification. Proteins having tyrosines 3–4 Å further away from the active site, or having active sites of lower reduction potential, may be more appropriate for further study.

Acknowledgment. We thank the SERC for postdoctoral support (to K.G. and N.P.T.), the SERC Molecular Recognition Initiative for Molecular Graphics Facilities, Dr. N. D. Wood for assistance with the pulse radiolysis experiments, Professor A. Holm in the University of Copenhagen for access to HPLC facilities, and Professor J. Ulstrup for most helpful discussion. We are also grateful to Dr. D. N. Beratan and colleagues at Cal Tech for supplying the pathways program.

Supplementary Material Available: Figures 1, 4, and 6 and their captions, showing an elution profile by FPLC on the reaction mixture from the NO_2 modification of parsley plastocyanin, the HPLC separation of trypsin-digested products from band 3 of parsley $PCu^{II}Tyr62NO_2$, and UV–vis spectra of tryptic peptides from the chromatogram for $PCu^{II}Tyr62NO_2$ (4 pages). Ordering information is given on any current masthead page.

(40) Meade, T. J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 4353.
(41) Margalit, R.; Kostic, N. M.; Che, C. M.; Blair, D. F.; Chiang, H. J.; Pecht, I.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6554.
(42) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *36*, 6968; *Mol. Cryst. Liq. Cryst.* **1991**, *194*, 215.
(43) Jacobs, B. A.; Mauk, M. R.; Funk, W. D.; MacGillivray, R. T. A.; Mauk, A. G.; Gray, H. B. *J. Am. Chem. Soc.* **1991**, *113*, 4390.
(44) Jackman, M. P.; McGinnis, J.; Powls, R.; Salmon, G. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1988**, *110*, 5880.
(45) Beratan, D. N.; Onuchic, J. N.; Gray, H. B. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1991; Vol. 27.