Rapid Intramolecular Electron-transfer in Pulse Radiolysis Experiments on the NO_2-modified Tyrosine83 Derivative of Plastocyanin

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The observation of an $NO_2^{-\bullet}$ radical intermediate in the pulse-radiolytic reduction of spinach plastocyanin PCu^{I}, NO_2-modified at Tyr83, but not in the case of the PCu^{II} derivative, is consistent with a rapid $NO_2^{-\bullet} \rightarrow Cu^{II}$ intramolecular electron-transfer process with $k > 10^7\ s^{-1}$.

Plastocyanin is a single (type 1) blue Cu protein involved in photosynthetic electron transport.[1] The chemical modification and procedure described define a new approach to metalloprotein electron-transfer studies, a particular aim of which is to understand better intramolecular effects.[2—5] Two regions on the surface of plastocyanin, M_r 10 500, 99 amino acids, have been identified as relevant to bimolecular electron-transfer reactions of the protein. One of these, the remote Tyr83 site near to negatively charged residues at 42—45, is used by positively charged inorganic and metalloprotein reactants. The other, the adjacent (to the Cu) His87 site, is less selective between different reactants.

Previously, in studies on nitro-phenolate complexes of the kind $[Co(NH_3)_5O_2C\text{–}X\text{–}PhNO_2]^{2+}$, where X = CH_2, CH=CH, and other groups of varying complexity, Hoffman and colleagues[6] have demonstrated that a nitro-radical derivative absorbing at ~300 nm can be generated by reduction with e_{aq}^-, $CO_2^{-\bullet}$ or $Me_2\dot{C}OH$ using pulse-radiolysis. Subsequently intramolecular electron-transfer from the radical $NO_2^{-\bullet}$ to the Co^{III} is observed. Rate constants for these mainly non-rigid systems, in which the separation of the $NO_2^{-\bullet}$ and Co^{III} is uncertain, and a rigid Co^{III}_2 system, are in the range 1—10^5 s^{-1}.[6,7] The reduction potential for the phenolate $NO_2^{-\bullet}$ radical has been determined as ~ −400 mV.[8]

The single NO_2-modified Tyr83 derivative of spinach plastocyanin has been prepared by reacting plastocyanin with tetranitromethane, $C(NO_2)_4$.[9—12] The protein was purified first by Sephadex G25-150 column chromatography, and then on a Pharmacia FPLC Mono Q column. The product in both the PCu^{I} and PCu^{II} states has a peak at 355 nm (ε 3400 $M^{-1}\ cm^{-1}$) attributed to the nitro group, which from known organic reactivity is assumed to be *ortho* to the phenolic group. Confirmation of a single NO_2-modification at Tyr83 has been carried out by peptide mapping.[12] The pK_a of the NO_2-modified Tyr83 is 8.1 as compared to 10.1 for native protein.[13] The reduction potential is within 25 mV of that for native protein.

Standard pulse-radiolysis procedures were used. Each pulse had a reducing capacity <10% of the total protein concentration, 9—49 μM. The pH was 7.0 (40 mM phosphate). In experiments using PCu^{II} Tyr83 NO_2, with sodium formate (12 mM) to generate $CO_2^{-\bullet}$ (−2.0 V), I = 0.10 M, the first pulse gave no evidence for $NO_2^{-\bullet}$ formation, peak at 300 nm (ε $10^4\ M^{-1}\ cm^{-1}$). Reduction of the $Cu^{II} \rightarrow Cu^{I}$ could be monitored at 597 nm (ε 4500 $M^{-1}\ cm^{-1}$), giving k = 5.7 × $10^8\ M^{-1}\ s^{-1}$ at 20 °C. This compares with a value 3.0 × 10^8 $M^{-1}\ s^{-1}$ obtained for native PCu^{II}; see Table 1 for summary. Subsequent pulsing gives increasing amounts of $NO_2^{-\bullet}$ with correspondingly less Cu^{II} reduction. This is assigned to the reduction of the nitro group of $PCu^{I}Tyr83NO_2$, which is the product of previous pulses.

One possible contributing factor is that $CO_2^{-\bullet}$ may not be a very effective reductant at the Tyr83 site. In further experiments, we therefore used positively charged Cd^+ as reductant (estimated reduction potential −1.80 V),[14] which like $CO_2^{-\bullet}$ is generated rapidly in the pulse radiolysis, in this case by reaction of e_{aq}^- with 100 μM Cd^{2+} in the presence of 10^{-2} M Bu^tOH to scavenge $OH^\bullet$ radicals. Again there is no production of $NO_2^{-\bullet}$ in the first pulse, unless small amounts of Cu^{I} protein happen to be present initially in the protein sample. The rate constant for $Cu^{II} \rightarrow Cu^{I}$ reduction, 4.9 × $10^9\ M^{-1}\ s^{-1}$, compares with 1.5 × $10^9\ M^{-1}\ s^{-1}$ for native protein. On further pulsing the generation of increasing amounts of $NO_2^{-\bullet}$ is observed.

In experiments with the reduced protein $PCu^{I}Tyr83NO_2$ present initially, formation of $NO_2^{-\bullet}$ was observed (k 4.2 × $10^9\ M^{-1}\ s^{-1}$) as the prime process occurring in the first pulse. We conclude therefore that the NO_2 group of modified Tyr83 is reduced in the first phase of reduction of both the Cu^{I} and Cu^{II} NO_2-modified proteins. An important difference in the case of the Cu^{II} protein is that reduction is followed by rapid transfer through to the Cu^{II} site. In the latter no $NO_2^{-\bullet}$

Table 1. Summary of rate constants (20 °C) at pH 7 (phosphate) for the reduction of spinach plastocyanin and the Tyr83 nitro derivative, I = 0.10 M (NaO_2CH or Na_2SO_4).

Reaction	$k/M^{-1}\ s^{-1}$
$PCu^{II} + CO_2^{-\bullet}$	3.0×10^8
$PCu^{II}Tyr83NO_2 + CO_2^{-\bullet}$	5.7×10^8
$PCu^{II} + Cd^+$	1.5×10^9
$PCu^{II}Tyr83NO_2 + Cd^+$	4.9×10^9
$PCu^{I}Tyr83NO_2 + Cd^+$	3.0×10^9

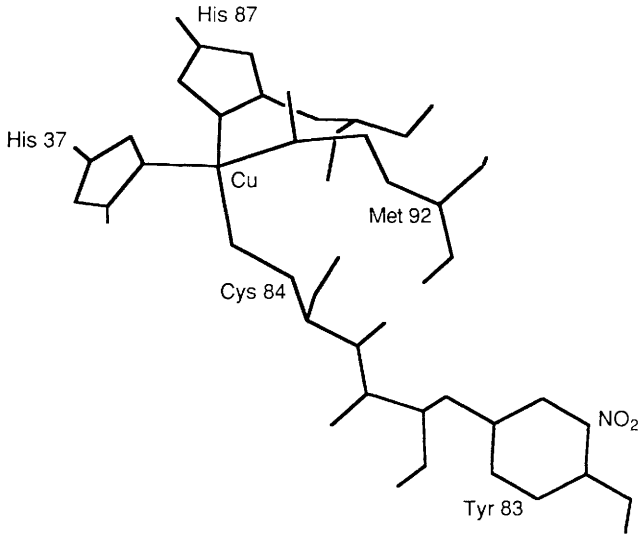

Figure 1. Computer graphics illustrating through-bond electron transfer route from Tyr83.

transient radical formation is observed, and any process involving $NO_2^{-\cdot}$ formation and decay is therefore extremely rapid, with the decay estimated at $>10^7$ s^{-1}. The three-fold faster rate constant for Cd^+ reduction of the Cu^{II} of $PCu^{II}Tyr83NO_2$ as compared to native protein indicates a significant supplement to electron transfer from reduction of the Cu^{II} *via* the remote site of the NO_2-modified protein. In the case of $PCu^{I}Tyr83NO_2$ the $NO_2^{-\cdot}$ decays by a slow intra- or inter-molecular process to another site or species which has not yet been identified. For a solution with $[PCu^{I}Tyr83NO_2]$ = 13.2 μM the decay on the first pulse occurs over 800 ms, but the site is saturated after 3—4 pulses, *i.e.*, ~30% reduction. In experiments on the Cu^{II}-containing protein, when this saturation is complete, a slow intermolecular reduction of Cu^{II} is observed.

The very fast intramolecular rate constant determined in this work is of interest since the NO_2 group of the modified Tyr83 lies on an electron-transfer path *via* Cys84 to the Cu. The through-bond route obtained by computer graphics, using data reported in the poplar plastocyanin crystal structure,[15] is illustrated in Figure 1. The distance from the nearest point on the tyrosine ring to the S(Cys), 7 bonds, is 10.7 Å, and the driving force ~770 mV. Of interest is an analogous route, HisCys, for intramolecular electron-transfer between the single (type 1) Cu site and the trimeric Cu site in the multi-copper enzyme ascorbate oxidase.[16]

This work is currently being extended to include the NO_2-modification of other tyrosines. The procedure described has potential for exploring the longer range of electron-transfer processes in metalloproteins.

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