

It was found that eight equivalents (per octamer) of reductant are consumed in the first stage followed by four equivalents in each of stages 2 and 3. These results were confirmed using the strong reductant  $[\text{Cr}(\text{edta})]^{2-}$  ( $E^\circ -1.0 \text{ V}$ ) which enabled spectra to be recorded more precisely at the end of each stage.

Reduction of metmyohemerythrin also occurs in three stages at pH 8.2. The first stage gives a first-order dependence on reductant. The rate constants for stages 2 and 3 ( $4.0 \times 10^{-3}$  and  $9.0 \times 10^{-4} \text{ s}^{-1}$  respectively) show no dependence on the nature or concentration of the reductant. Further detail of these different stages will be presented.

The existence of a quarter-met intermediate in the reduction of methemerythrin (product of the second stage) indicates that the Fe(II,III) units generated at the end of the first and second stages are not identical. Furthermore, comparison of the rate constants for the second stage of the reduction of methemerythrin and metmyohemerythrin suggests a common rate-controlling conformational change in both cases.

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PS2.25 — MO

J.D. SINCLAIR-DAY

A.G. SYKES

Department of Inorganic Chemistry

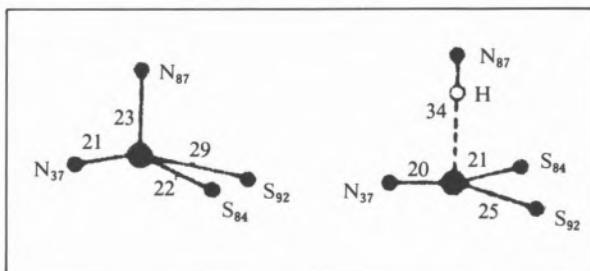
The University

Newcastle upon Tyne

England

## ACID DISSOCIATION CONSTANTS FOR THREE PLASTOCYANINS

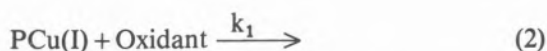
The metalloprotein plastocyanin (M.Wt. 10,500) consists of 99 amino acids and a single (Type 1) Cu active site [1]. It is involved in electron transport ( $E^\circ \sim 360\text{mV}$ ) in the chloroplast where it functions as an oxidant for cytochrome *f* and a reductant for P700. The Cu atom is coordinated by His37, Cys84, His87 and Met92 (NSNS donor atom set) in a distorted tetrahedral arrangement for both Cu oxidation states. Proton induced deactivation of the active site [2] has been shown to correspond to dissociation of His87 yielding a redox-inactive three-coordinate structure [3].



The acid dissociation constant,  $K_a$ , can be determined using NMR



spectroscopy [4] which allows direct observation of the relevant protonation. The kinetic method employs oxidants such as  $[\text{Fe}(\text{CN})_6]^{3-}$  and  $[\text{Co}(\text{phen})_3]^{3+}$  to probe the reactivity of the protein in the pH range of interest, where PCu(I) but not HPCu(I) is redox active (2).



The dependence (3) enables  $K_a$  to be determined.

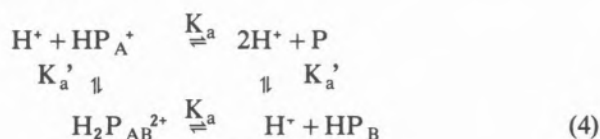
$$k(\text{M}^{-1}\text{s}^{-1}) = \frac{k_1 K_a}{[\text{H}^+] + K_a} \quad (3)$$

Kinetic determination of  $\text{pK}_a$ 's using  $[\text{Fe}(\text{CN})_6]^{3-}$  as an oxidant are in satisfactory agreement with those by NMR. However, the use of  $[\text{Co}(\text{phen})_3]^{3+}$  yields  $\text{pK}_a$ 's which are consistently higher, see Table.

Table  
Experimentally determined  $\text{pK}_a$ 's for different plastocyanins

Source	NMR	$[\text{Fe}(\text{CN})_6]^{3-}$	$[\text{Co}(\text{phen})_3]^{3+}$
Parsley	5.7	5.5	6.1
Spinach	4.9	4.9	5.7
<i>A. variabilis</i>	5.1	4.8	5.5

The results may be explained by introducing a second protonation effective in the oxidation by  $[\text{Co}(\text{phen})_3]^{3+}$  as in (4)-(6). Sites for the two independent protonations are designated A and B where A refers to the active site protonation at His87.

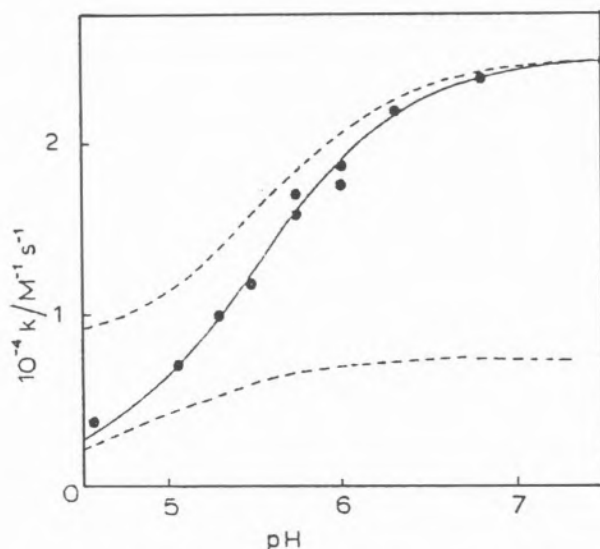


This scheme gives (7)

$$k(\text{M}^{-1}\text{s}^{-1}) = \frac{k_1 K_a K_a' + k_2 K_a [\text{H}^+]}{K_a K_a' + K_a' [\text{H}^+] + K_a [\text{H}^+] + [\text{H}^+]^2} \quad (7)$$

The figure shows the best fit for spinach PCu(I) using  $K_a$  as determined by NMR, and indicates the component profiles arising from each protonation. This procedure gives  $\text{pK}_a'$  values for parsley (5.8), spinach (5.7) and *A. variabilis* (5.7).

The effect of  $K_a'$  is consistent in simple electrostatic terms with a proton associating at, or near, the site of electron transfer for a positively charged oxidant. The involvement of  $K_a'$  in the oxidation by  $[\text{Fe}(\text{CN})_6]^{3-}$  is small or negligible indicating



that oppositely charged complexes use different binding locations on plastocyanin. These conclusions are consistent with earlier NMR studies [5,6] which define binding sites close to His87 and Tyr83 for  $[\text{Fe}(\text{CN})_6]^{3-}$  and  $[\text{Co}(\text{phen})_3]^{3+}$  respectively. We therefore propose the protonation corresponding to  $K_a'$  is at the binding site near to Tyr83.

The  $\text{pK}_a'$  value of  $\sim 5.7$  is higher than expected for a single carboxylate residue but may be explained in terms of two carboxylates sharing one proton. There are a number of carboxylate residues close to Tyr83, notably the highly conserved 42-45 residues in plant plastocyanins, also Asp42 and Glu85 in *A. variabilis* plastocyanin. These could provide an adequate site for protonation and association of  $[\text{Co}(\text{phen})_3]^{3+}$ . The amino-acid sequence for parsley plastocyanin indicates deletions at 58 and 59 which could explain the higher  $\text{pK}_a$  value.

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PS2.26 — TH

M.E. SANDER

H. WITZEL

Institute of Biochemistry  
University of Münster  
F.R.G.

### CARBOXYPEPTIDASE A, EVIDENCE FOR AN ANHYDRIDE INTERMEDIATE

In the reaction of carboxypeptidase A, a  $Zn^{2+}$  containing exopeptidase, it is still in discussion, whether Glu-270 forms a covalent anhydride intermediate with the acyl group of the substrate or is involved in a general base catalysis mechanism. Evidence for an acyl enzyme has been obtained at  $-60^{\circ}C$  only spectroscopically with one ester substrate, the *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate (Makinen).

Since it should be possible to trap such an anhydride intermediate with nucleophiles under conditions where accumulation can be expected, we incubated the enzyme at  $-75^{\circ}C$  with several hippuric esters or -peptides in the presence of hydroxylamine and *O*-methylhydroxylamine. Thus the enzyme could be inactivated, while it was still active in the blank probe with the same concentration of hydroxylamine. Inactivation was most efficient with hippuryl glycolic acid, a relatively slow substrate for the carboxypeptidase A. A hydroxamate group within the protein could be identified by the visible spectrum of its  $Fe^{3+}$ -complex, which was characteristic for that of a glutamic acid. Such a hydroxamate group, responsible for the inactivity of the enzyme and produced only in the presence of a substrate, can be expected, if this glutamic acid forms an anhydride intermediate. Furthermore we could stabilize the acyl enzyme by adding denaturing agents at  $-60^{\circ}C$  during the reaction with hippuryl-L-phenylalanine or *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactate. This loaded enzyme was reduced with  $[^3H]NaCNBH_3$ . After complete hydrolysis of the protein the  $[^3H]$   $\alpha$ -ami-

no- $\delta$ -hydroxyvaleric acid, which is formed as the reduction product of an acylated glutamic acid, could be identified by high voltage paper electrophoresis and paper chromatography. These results can also be interpreted only on the basis of an anhydride intermediate.

A tryptic digest of the reduced protein gives evidence that the glutamic acid in question is Glu-270.



PS2.27 — TH

JUNZO HIROSE

MASAHIDE NOJI

YOSHINORI KIDANI

Faculty of Pharmaceutical Sciences  
Nagoya City University  
Tanabe-dori 3-1, Mizuho-ku, Nagoya 467  
Japan

RALPH G. WILKINS

Department of Chemistry  
New Mexico State University  
Las Cruces, N.M. 88003  
U.S.A.

### THE INTERACTION OF ZINC IONS WITH ARSANILAZOTYROSINE-248-CARBOXYPEPTIDASE A

Arsanilazotyrosine-248-carboxypeptidase A([(Azo-CPD)Zn]) [1] is a derivative modified with chromophoric arsanilazotyrosine-248 residue. [(Azo-CPD)Zn] has almost the same activity as native carboxypeptidase A. It has proven particularly useful for studying the conformation of the enzyme [2] and metal binding to the apo-enzyme [3]. At pH 7.5-8.8, the arsanilazotyrosine-248 residue forms an intramolecular complex with the zinc ion at the active site and gives a characteristic optical absorption at 510 nm.

When excess zinc ions were added to [(Azo-CPD)Zn], the characteristic red color, which arose from the intramolecular complex of the arsanilazotyrosine-248 residue with the active