# Identification of Binding Sites in Reactions of Blue Copper Proteins with Inorganic Complexes and Implications of Such Findings

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*Summary* Kinetically determined p$K_a$ values provide evidence for different protein binding sites; implications regarding the application of Marcus theory and calculation of protein self-exchange rate constants are considered.

AZURINS (M. W. *ca.* 14,600, 128 amino acids) are blue copper proteins from bacterial sources, which like plastocyanins (M.W. *ca.* 10,500, 99 amino acids) have a single copper atom.[1,2] Both protein types function as electron carriers using the $Cu^{I}$ and $Cu^{II}$ oxidation states, which, from a variety of physical measurements[3,4] and *X*-ray crystal structure determinations of the $Cu^{II}$ states,[5,6] are now known to be co-ordinated to two histidines, a cysteine, and a methionine. A number of properties indicate close similarities between azurins and plastocyanins and homologies in the peptide chains have been noted.[6] One difference is that the azurins have two additional histidines (Nos. 35 and 83) not bonded to the Cu, whereas plastocyanins have no histidines additional to the two bonded to the Cu.

Azurin from a culture of *Pseudomonas aeroginosa*, u.v.–visible absorbance $A_{280}/A_{625}$ ratio of 1·67 (lit.,[7] 1·72), was used in these studies. Although reactions of azurin with inorganic complexes as redox partners have been investigated previously,[8] in only one case, the $[Fe(edta)]^{2-}$ ($H_4$edta = ethylenediaminetetra-acetic acid) reduction of $ACu^{II}$, has a pH dependence been reported. For the pH range 5·0—9·1, $I = 0{\cdot}10$ M (NaCl), the buffers cacodylate, phosphate, and borate ($10^{-2}$ M) were used without any significant differences in regions of overlap. Individual runs with the complex in > 10-fold excess of the protein (*ca.* $1 \times 10^{-5}$ M) gave good first-order kinetics, from which second-order rate constants, *k*, could be obtained in a straightforward manner providing the concentration of complex was kept sufficiently small as to exclude limiting kinetic behaviour.[9]

Rate constants (25 °C) for the oxidation of azurin $ACu^{I}$ with $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and $[Fe(CN)_6]^{3-}$ (see Figure), yield protein acid dissociation p$K_a$ values of 7·5 and 7·1 respectively. With $[Co(4,7\text{-DPSphen})_3]^{3-}$† as oxidant little or no pH dependence was observed in the range 6·3—9·0 investigated. Although the reaction of $ACu^{II}$ with $[Fe(CN)_6]^{4-}$ is thermodynamically slightly unfavourable it was possible to study this reaction by having the reductant in sufficient excess, when a p$K_a$ of 6·1 was obtained; *cf.* 6·4 for $[Fe(edta)]^{2-}$.

Histidine is the only amino acid which in the free state is protonated at pH *ca.* 7. From n.m.r. studies the two histidines not bonded to the Cu give p$K_a$ values of 7·5 and 7·1.[10] The first of these p$K_a$ values is not markedly affected by the oxidation state of the Cu, and shifts to 7·3 for $ACu^{II}$, unlike the p$K_a$ for the second histidine which exchanges protons more slowly and is believed to be closer to the Cu. The kinetic p$K_a$ values are seen to be in close agreement with n.m.r. values. Therefore, different binding sites can be defined for $[Co(phen)_3]^{3+}$ (influenced by Hist-83) and the $[Fe(CN)_6]^{3-,4-}$ couple (influenced by Hist-35). We note that the reaction of the much larger oxidant $[Co(4,7\text{-DPSphen})_3]^{3-}$ is not affected by either p$K_a$, suggesting that the reaction is not influenced by either histidine. Furthermore the oxidant $[Co(phen)_3]^{3+}$ does not appear to respond to the p$K_a$ of 7·1 and *vice-versa* for $[Fe(CN)_6]^{3-}$, the implication being that the two binding sites are well separated in the protein.

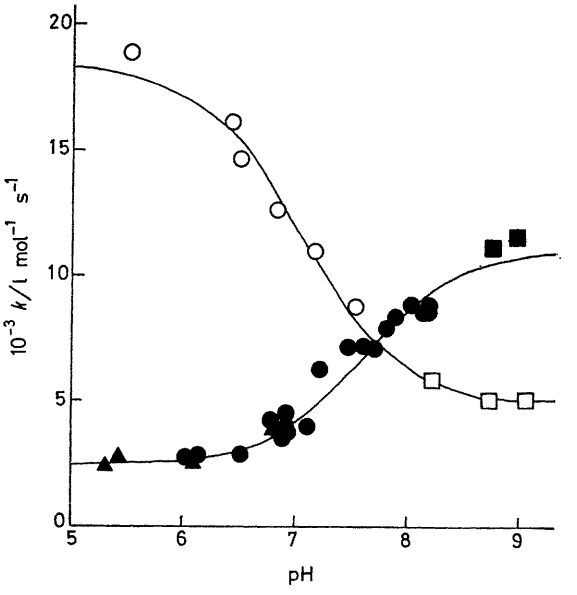


FIGURE. The variation of second-order rate constants, *k*, with pH for the oxidation of *P. aeroginosa* azurin, $ACu^{I}$, with $[Co(phen)_3]^{3+}$ (filled symbols) and $[Fe(CN)_6]^{3-}$ (open symbols) at 25 °C, $I = 0{\cdot}10$ M (NaCl), with $10^{-2}$ M phosphate (●,○), borate (■,□), and cacodylate (▲) buffers.

For reactions of parsley plastocyanin, $PCu^{I}$, pH effects are also observed with $[Co(phen)_3]^{3+}$ (p$K_a$ 6·1) and $[Fe(CN)_6]^{3-}$ (p$K_a$ 5·7).[11] Assignment of these p$K_a$ values is less straightforward where they could arise from protonation of glutamate or aspartate. A striking feature of the profiles is that the rate constants decrease with pH to give at pH < 5 a switch-off in reactivity. Since $[Co(4,7\text{-DPSphen})_3]^{3-}$ does not respond to pH 5·2—7·5, clearly dissociation at the $Cu^{I}$ is unlikely to provide an explanation. As an alternative, it is possible that protonation induces a conformation change affecting the $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ binding sites, where these are in the same locality on the protein. No similar effect of $H^+$ on the reactivity of $PCu^{II}$ is observed.

† 4,7-DPSphen is an abbreviation for 4,7-di(phenyl-4′-sulphonate)-1,10-phenanthroline ≡ 4,7-bis-(4-sulphophenyl)-1,10-phenanthrolinato(2−).

It is known that rate constants for a protein-complex redox reaction are the product of $K$ for prior association, and $k_{et}(s^{-1})$ for electron transfer within the adduct. Protein reduction potentials are often determined against an inorganic redox couple.[12] Thus, from our studies with the $[Fe(CN)_6]^{3-,4-}$ couple, it can be concluded that the potential for azurin decreases from 360 mV at pH 5·0 to 300 mV at pH 9·1, and the potential for plastocyanin increases with decreasing pH from 380 mV.[11] In contrast, the potentials determined using the $[Co(4,7\text{-DPSphen})_3]^{3-}$ couple will probably be invariant with pH.

The existence of different binding sites (where the separation of the Cu from the redox partner will vary as the reaction site changes) and also different reduction potentials (which determine calculated equilibrium constants for the overall redox process) is of considerable interest. For two inorganic complexes Marcus theory for outer-sphere electron-transfer is known to provide a satisfactory approach and leads to self-exchange rate constants which correspond reasonably well with measured values.[13] For protein-complex reactions no unique protein self-exchange rate constant can be obtained in the same way. Even when two complexes use the same site, the same self-exchange value can only result if the association constants $K$ are similar and electrostatic in origin. It is now known[11] that in some reactions at least, *e.g.* $ACu^{I} + [Fe(CN)_6]^{3-}$, other specific effects are influential, affecting the thermodynamic parameters $\Delta H^{\circ}$ and $\Delta S^{\circ}$ and hence $K$. Certainly the identification of at least two binding sites in these reactions of azurin and plastocyanin is not unreasonable in view of the electron transport function of the protein, and the need to transfer electrons in and out of the protein.

(*Received, 20th September* 1978; *Com.* 1018.)

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