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STEADY-STATE KINETICS OF CYTOCHROME *c* OXIDASE IN PHOSPHOLIPID VESICLES: THE EFFECTS OF pH, IONIC STRENGTH AND D₂O

The steady-state kinetics of cytochrome *c* oxidase (E.C. 1.9.3.1) reconstituted into phospholipid vesicles has been investigated with a spectrophotometric method, using the high activity region of the reaction between reduced cytochrome *c* and the enzyme. k_{cat} and k_{cat}/K_m were evaluated at high ionic strength ($I=0.5$) in the pH range 5.2-8.6, and the effects of varied ionic strength and D₂O were studied in a more limited pH range. Throughout the investigation, the zwitterionic buffers Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid] and Mes [2-(*N*-morpholino) ethane sulfonic acid] were used at a concentration of 0.05 M. The ionic strength was changed by inclusion of various amounts of K₂SO₄. The orientation was found to be 65% cytochrome *a* on the outside of the vesicle. Consequently, 65% of the total concentration was used when the kinetic parameters were calculated.

Principal results and conclusions are as follows: At high ionic strength ($I=0.5$) k_{cat} increases with decreasing pH from 35 s⁻¹ at pH 8.6 to 1000 s⁻¹ at pH 5.2. This result is qualitatively in accordance with one earlier study, with the enzyme in detergent solution, whereas most other investigators have reported a pH maximum for k_{cat} . The pH dependence of k_{cat} cannot be simulated with less

than three pK_a values, which suggests at least three sites, each increasing the activity on protonation. Our estimated pK_a values (7.8, 6.8 and 4.5) are within experimental uncertainty the same as those found in detergent solution.

Variation of the ionic strength from $I=0.05$ to $I=0.5$ has little effect on k_{cat} but k_{cat}/K_m , and thus K_m , is a function of ionic strength. The velocity at a given cytochrome *c* concentration generally decreases with increasing ionic strength. At low pH (6.2) the effect seems to be more complicated as our results show a maximum of k_{cat}/K_m around $I=0.25$. k_{cat}/K_m was essentially constant at high ionic strength ($I=0.5$) in the pH range 5.2-8.6, i.e. K_m also increases with decreasing pH. This suggests that the pH effect is mainly on the rates of catalytic steps, rather than on the combination between enzyme and substrate. It is not obvious which particular steps are affected.

The effects of D₂O were studied in the pD range 5.8-7.2. Taking into account both the difference between pH and pD and the D₂O-induced shift in pK_a ($pK_a^D = pK_a^H + 0.54$) we found that D₂O decreases k_{cat} by a factor of about 2. k_{cat}/K_m is, on the other hand, approximately the same in H₂O and D₂O. From the D₂O-data we conclude that an intramolecular proton transfer step is at least partially rate limiting. This contradicts the common assumption that the product dissociation step of the reaction is rate limiting.

Based on our results and a minimal mechanism involving 13 consecutive steps in the catalytic cycle of cytochrome oxidase, we argue that an intramolecular step which involves both electron and proton transfer is rate limiting. The step is suggested to be important in the function of cytochrome oxidase as a proton pump.



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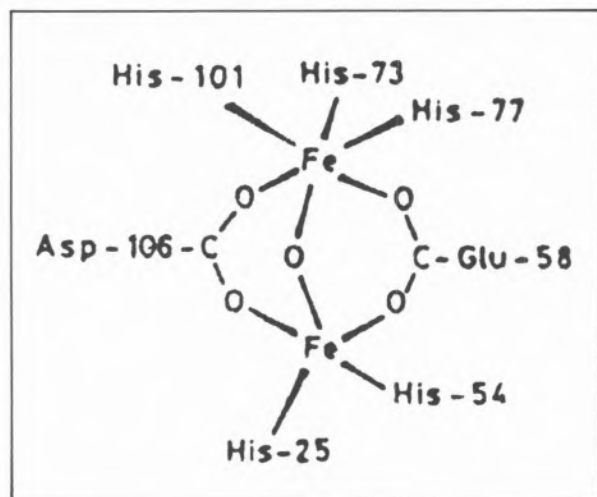
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ACTIVE SITE CHEMISTRY OF OCTAMERIC AND MONOMERIC HEMERYTHRIN FROM *THEMISTE ZOSTERICOLA*

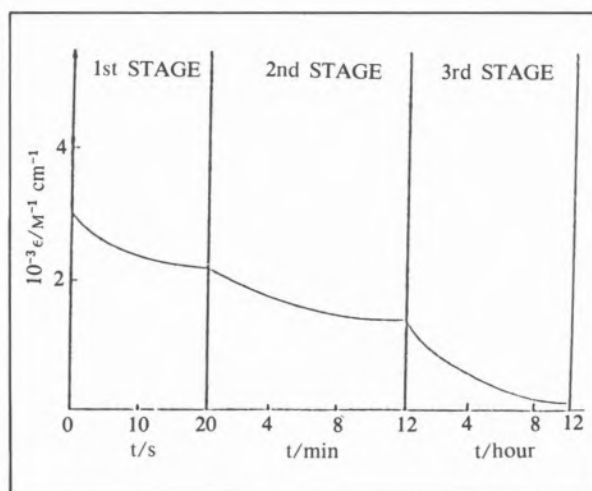
Hemerythrin [1,2] was obtained from the coelomic fluid of *Themiste Zostericola* as the octamer (M.Wt. 108,000), which has eight identical subunits, and from the retractor muscle as the monomer, metmyohemerythrin (M.Wt. 13,900). Each subunit is known to contain a binuclear Fe active site capable of binding O_2 .

The structure of the active site of the met, Fe(III,III), form is as shown [3]. Coordination of hydroxide (corresponding to an acid dissociation of pK_a ca. 8.0) is known to occur at the five-coordination iron. Using EXAFS [4] it has been demonstrated that the deoxy, Fe(II,II), form has no μ -oxo bridge and there is an increase in the Fe-Fe distance.



Reduction of octameric methemerythrin has been studied by WILKINS *et al.* using dithionite and a semi-met, $Fe(II,III)_8$, intermediate has been successfully characterised. It has also been proposed that further reduction proceeds by long-distance intramolecular electron-transfer (ca. 30 Å yielding $Fe(II,II)$ and $Fe(III,III)$ subunits [5], the latter being rapidly re-reduced to $Fe(II,III)$.

To further augment this study, and monitor precisely the latter stages of reduction, detailed investigations of the reduction of methemerythrin and metmyohemerythrin using as one-electron reductants the Sargeson cage complexes $[Co(sep)]^{2+}$ ($E^\circ -0.30$ V) and $[Co(sarCl_2)]^{2+}$ ($E^\circ -0.13$ V), the triazacyclononane complex $[Co(9-aneN_3)]^{2+}$ ($E^\circ -0.40$ V) and $[Cr(bipy)_3]^{2+}$ ($E^\circ -0.26$ V) have been carried out. Three stages are clearly seen in the reduction of methemerythrin (pH 6.3 - 9.0). The figure shown is for a reaction monitored at 400 nm with $MetHr = 1.0 \times 10^{-4}$ M (expressed as monomer), $[Co(sep)]^{2+} = 1.4 \times 10^{-3}$ M, pH 6.3, $I = 0.15$ M (Na_2SO_4). The rate of the first stage shows a first-order dependence on reductant. Rate constants for stages 2 and 3 (3.7×10^{-3} and $1.2 \times 10^{-4} s^{-1}$ respectively, pH 8.2) show no dependence on the concentration or nature of the reductant consistent with an intramolecular process. Hydrogen-ion dependencies are observed for the first and second stages. Rate constants for stage 3 are however independent of $[H^+]$.



Consumption of reductant in the different stages was determined using $[Cr(phen)_3]^{2+}$ which has an intense absorbance at 850 nm ($\epsilon = 6500$ $M^{-1} cm^{-1}$).