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Review

The steady-state kinetics of cytochrome *c* oxidation by cytochrome oxidase

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Contents

I. Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*: O_2 oxidoreductase; EC 1.9.3.1) is the terminal enzyme in the mitochondrial electron transport chain. It catalyzes the oxidation of ferrocytochrome *c* to ferricytochrome *c*

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; DCCD, *N,N′*-dicyclohexylcarbodiimide; RCR, respiratory control ratio.

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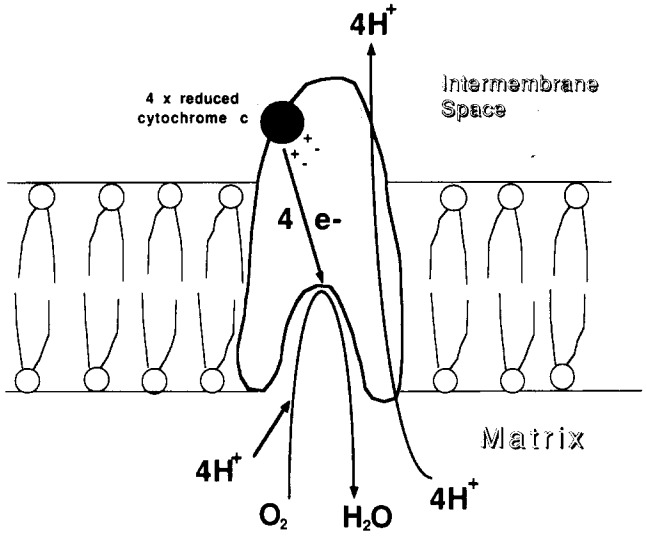

Fig. 1. A model of cytochrome oxidase function.

and the consequent reduction of oxygen to water. In the process it generates a proton electrochemical potential across the inner mitochondrial membrane, which is later utilized to drive the thermodynamically unfavourable synthesis of ATP. In the model shown in Fig. 1 this potential is generated by a combination of the movement of electrons from ferrocytochrome *c* to the oxygen reduction site [1] and the translocation of protons from the matrix to the intermembrane space [2].

Cytochrome oxidase contains two spectrally distinct haem *a* centres [3] – *a* and a_3 – the former usually being of low spin and not reacting with ligands and the latter usually being high spin and binding O_2 and CO in the ferrous state and HCN, HN_3 and H_2S in the ferric state. Two [4] or possibly three [5] copper atoms are also an integral part of the electron transfer mechanism of cytochrome oxidase. They are named Cu_A, Cu_B and Cu_X – the latter being only recently discovered and currently of disputed stoichiometry (compare Ref. 5 with Ref. 6) and unknown function. The bovine enzyme also contains 1 zinc and 1 magnesium atom per aa_3 [6,7]. Prokaryotic aa_3-type cytochrome oxidases are present in the cell membranes and consists of two or three polypeptide subunits only [8,9]. The subunit structure of eukaryotic cytochrome aa_3-type oxidases is more complex; the yeast enzyme contains nine subunits and the mammalian enzyme up to thirteen [10,11]. The bovine enzyme has a molecular mass of approximately 200 kDa/monomer, though it can also exist as a dimer – the relative importance and function of the two forms being a matter of debate [12–16].

All cytochrome aa_3-type oxidases are able to catalyze the oxidation of ferrocytochrome *c*. The physiological substrate for the eukaryotic enzymes is a soluble, basic cytochrome *c*. The physiological substrate for many of the prokaryotic enzymes is less clear [8]: some thermophilic bacteria (e.g., PS3) contain a covalently bound cytochrome *c*-aa_3 complex that is catalytically competent [17]; other (e.g., *Paracoccus denitrificans*) are capable of oxidizing a membrane-bound cytochrome *c* in preference to, or in addition to, their own soluble acidic cytochrome *c* [8,18,19].

Whilst cytochrome *c* is one of the best characterized proteins, structurally and functionally [20], its interaction with cytochrome *c* oxidase is less well characterized. Studies on the steady state kinetics of cytochrome *c* oxidation can help to answer mechanistic questions about the transfer of electrons between the two proteins, as well as providing insights into physiological questions (e.g., control of enzyme turnover). Until recently many of these questions have been overshadowed by controversy over the experimental data. Now, however, a consensus has emerged as to the effects of substrate concentration, pH and ionic strength on the kinetic parameters and the debate has shifted to the models that can explain this data. This therefore appears an appropriate time to review the steady-state kinetic data for the oxidation of cytochrome *c* by cytochrome aa_3-type oxidases, and critically examine the competing models.

Most authors have studied the oxidation of horse heart cytochrome *c* by bovine cytochrome *c* oxidase (the bovine enzyme exhibits similar kinetics with its own cytochrome *c* [21]). However, useful information can also be obtained by comparing this data with that obtained from alternative oxidases and cytochromes *c*.

II. Time-course of cytochrome *c* oxidation

The first 'problem' with cytochrome oxidase kinetics has been the time-course of the oxidation of reduced *c*. Most enzyme kineticists diligently avoid measuring anything other than initial rates of reaction, because of the simplicity of the assumption that the product concentration is 0 [22]. Thus, the integrated rate equations and exponential time-courses beloved of physical chemists have been shunned. The 'problem' with cytochrome *c* oxidation is that it is too simple – it is strictly first order, even at higher cytochrome *c* concentrations [23,24]. Surprisingly, the first order rate constant is dependent on the total concentration of substrate *and* product (ferro- and ferricytochrome *c*) and Michaelis-Menten hyperbolic kinetics are obeyed whether velocity is plotted against [S] *or* [S + P]. As first shown by Henri [25] first-order kinetics should not be observed when an enzyme complex is formed because the integrated rate equation is of the form

$$-V_{max} \cdot t = ([S] - [S_0]) + (K_m \cdot \ln([S]/[S_0]))$$

In the initial part of the progress curve, [S] remains close to $[S_0]$ and the left hand linear term becomes negative faster. However, as the [S] becomes signifi-

cantly smaller than $[S_0]$, the logarithmic term starts to become more important. Thus, idealized time-courses of reaction mechanisms involving enzyme-substrate complexes (with no product inhibition) tend to show an initial linear decrease of [S] with time, which then becomes increasingly exponential [20].

Reasoning on the above lines, Smith and Conrad [23] took the first-order kinetics at face value and presumed that the reaction proceeded via a bimolecular collision mechanism with no enzyme-substrate complex formation. However, a first-order rate constant should remain unchanged with increasing cytochrome *c* concentration To explain the decrease actually observed, Smith and Conrad had to postulate that the hyperbolic dependence of the initial velocity on cytochrome *c* concentration was due to inhibition of the reaction by cytochrome *c* itself. In favour of such an analysis is the fact that the *P. denitrificans* cytochrome oxidase respiring on soluble Paracoccus (but not bovine) cytochrome *c*-550 does not exhibit saturation kinetics, i.e., the rate of cytochrome *c* oxidation increases continually with increasing substrate concentration [26]. This is in contrast to most enzymes forming a Michaelis enzyme-substrate complex where enzyme turnover reaches a maximum (V_{max}) at a finite substrate concentration (although see catalase for a notable exception [27]). However, the fact that under some conditions deviations have been reported from first-order oxidation kinetics is evidence against the bimolecular collision mechanism [28]. Combined with the evidence that both ferro- and ferricytochrome *c* form a stable [29] and sometimes covalent [17] complex with cytochrome oxidase, an explanation of the first-order kinetics has been sought that would not abandon the idea of an enzyme-substrate complex. Minnaert [24] proposed that if the rate equation is of the form:

$$v = \frac{\text{Constant} \cdot [S]}{(\text{Constant} \cdot [P+S]) + \text{Constant}}$$

and [P + S] is constant, then $v = \text{Constant} \cdot [S]$ and the reaction has a first-order time course. Michaelis-Menten kinetics are also obtained if [S] or [P + S] are varied. However, in order to produce a steady-state rate equation of this form, Minnaert showed in his mechanism IV that the reaction must be of the form:

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} ES \underset{k_2}{\rightarrow} EP \underset{k_3}{\overset{k_{-3}}{\rightleftarrows}} E + P$$

and that $k_1 = k_{-3}$ and $k_{-1} = k_3$, i.e., the enzyme cannot distinguish between substrate and product. Thus this predicts that the on and off rates for bound ferro- and ferricytochrome *c* must be identical. In addition, internal electron transfer (k_2) must be irreversible. This can either be achieved by presuming that k_2 is very large, or that the reverse reaction (k_{-2}) is very small. It is important to realize that the Minnaert mechanism does not require that the rate of internal electron transfer must be fast, as some authors have suggested [29], only that it must be a lot faster than the reverse rate. Given this mechanism:

$$K_m(\text{for } P+S) = \frac{k_{-1}}{k_1} \text{ and } V_{max} \text{ for } [P+S] \rightarrow \infty = \frac{k_{-1} \cdot k_2}{(k_{-1} + k_2) \cdot \left(1 + \frac{P}{S}\right)}$$

Therefore, K_m is equivalent to the dissociation constant for cytochrome *c* and, depending on the conditions, either internal electron transfer [30,31] or product dissociation [32,33] can limit the rate of turnover at saturating cytochrome *c* concentrations.

Whilst the Minnaert mechanism has been gratefully accepted as a solution to the observed kinetics, it is important to remember two points; the first-order time-course may not exist under all conditions and thus should always be tested for; and secondly, the prediction that the enzyme cannot distinguish between the binding of ferro- and ferricytochrome *c* needs to be confirmed. Yonetani and Ray [28] have demonstrated that, under conditions where first-order kinetics are observed, the K_i for ferricytochrome *c* is the same as the apparent K_m for ferrocytochrome *c*. At more alkaline pH values, where the kinetics deviate from a first-order time-course, the two values diverge. However, aqueous two-phase partition studies at low ionic strength and neutral pH show that the affinity of the enzyme for ferricytochrome *c* is 3-times greater than that for the reduced form [34]. Furthermore, direct binding studies on mitochondria suggest that there is a difference in the dissociation constant between reduced and oxidized cytochrome *c* [35] and that this accounts for the difference in apparent redox potential between freen and bound cytochrome *c* [36]. However, some, but not all [37], of this difference can be accounted for by a lowering of the cytochrome *c* E_m upon binding to lipids [38].

Although these studies were performed under conditions where it was not possible to check directly whether first-order kinetics were obeyed, they still raise questions as to whether the Minnaert mechanism is universally correct.

III. Kinetic assays of cytochrome *c* oxidation rates

III-A. Polarographic and spectrophotometric assays compared

The second problem in cytochrome oxidase kinetics is that the reaction does not obey Michaelis-Menten kinetics at low ionic strength [23,39,40], i.e., non-linear Lineweaver-Burk or Eadie-Hofstee plots are obtained. Connected with this, the kinetics of the reaction are also

different depending on whether the initial rate of oxidation of horse ferrocytochrome *c* is monitored spectrophotometrically, or the steady-state reduction of oxygen is followed polarographically. Although both assays (see section IV) show biphasic Eadie-Hofstee plots with a 'high-affinity, low-turnover' phase and 'low-affinity, high-turnover' phase [40–42], the kinetic parameters with respect to cytochrome *c* are different. Under the same conditions of pH and ionic strength (25 mM Tris-acetate, pH 7.9) the spectrophotometric assay and the polarographic assay give the same high-affinity K_m, but the V_{max} for the polarographic assay is 5–10-times higher. The V_{max} of the low-affinity phase is generally about twice as large in the polarographic assay, though the K_m is normally also larger [42].

The reason for these differences is that the two assays are subject to different rate-limiting steps. The polarographic steady state assay normally employs ascorbate and TMPD as artificial electron donors to cytochrome *c*. Ascorbate (the ultimate electron donor) is kept in excess as the rate constant for its reduction of TMPD is very high. As the rate constant for cytochrome *c* reduction by TMPD is also high, the addition of TMPD overcomes earlier problems due to the reduction of cytochrome *c* by ascorbate becoming rate limiting [43]. Indeed cytochrome *c* can be shown to be 98% reduced in the steady-state [44]. However, TMPD can also reduce cytochrome *c* when it is bound to cytochrome oxidase – in fact the rate constant is higher for bound *c* ($1.3 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ for bound and $3.5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ for free [45]). This explains the difference between the two assays as the polarographic assay introduces a new step [46]:

$$E + S \rightleftarrows ES \rightarrow EP \rightleftarrows E + P$$

(TMPD; $TMPD^+$)

Thus, enzyme turnover can occur without the dissociation of bound product [44]. As the dissociation of bound ferricytochrome *c* has been shown to be rate-limiting for the spectrophotometric assay under some conditions [32,33], this explains why the V_{max} for the high affinity phase is higher in the polarographic assay (though the K_m values are similar). Indeed studies on the pre-steady-state reaction of TMPD with cytochromes *c* from a variety of organisms have confirmed that the 'rate-limiting step' in the polarographic assay is usually the reduction of bound cytochrome *c* by TMPD [46]. Although the above data may not hold for all conditions of pH, ionic strength and cytochrome *c* concentration, and the concept of a single rate-limiting step in a complex mechanism may be an oversimplification (cf. section IV-B.), it is important to remember that the two assays may not respond equally to different conditions. Thus, a reagent that increases the dissociation of bound cytochrome *c* would be expected to increase the activity as monitored by the spectrophotometric assay more than the polarographic assay. Indeed, low levels of poly(L-lysine) (which stimulate the release of cytochrome *c* from the cytochrome *c*-aa_3 complex [32]) behave exactly in this fashion when the Paracoccus oxidase is assayed with its own soluble cytochrome *c* [47].

III-B. Relevance of kinetic assays to the physiological state

As the rate-limiting step in the polarographic assay can involve artificial electron donors, many authors have concluded that the spectrophotometric assay must be more 'physiological' [48–50]. Although the absence of artificial electron donors makes it easier to model the spectrophotometric assay as the number of parameters is reduced [51], it does not necessarily follow that the spectrophotometric assay is more physiological. In particular, the assumption that the reduction of bound ferrocytochrome *c* does not occur in vivo is not proven. Although studies by Gupte et al. [52] using Fluorescence Recovery After Photobleaching (FRAP) suggest that the rates of electron transfer in vivo are low enough to allow cytochrome *c* to diffuse randomly from the bc_1 complex to cytochrome oxidase, other data suggest that it might be necessary to assume that the respiratory complexes form super-molecular aggregates [53,54]. Indeed, Nicholls and co-workers [29,55] have postulated that one molecule of cytochrome *c* is always bound to the oxidase in mitochondria. In this case it is possible that the bc_1 complex might reduce the cytochrome *c*-aa_3 complex in vivo, although as the interaction domains of the oxidase and the bc_1 complex for cytochrome *c* overlap [56,57] it is probable that some dissociation of the *c*-aa_3 complex must occur for *c* to be reducible by bc_1. Alternatively, free ferrocytochrome *c* might reduce the bound ferricytochrome *c*. At any rate, it is clear that the reduction of bound *c* must be physiological for the bacterium PS3, as in this case cytochrome aa_3 is covalently bound to its catalytic cytochrome *c* [17].

There is also evidence that a soluble cytochrome *c* may not be essential for cytochrome oxidase turnover in some species. *P. denitrificans* cytochrome aa_3 preferentially oxidizes a cytochrome *c* (*c*-552) that remains bound to the bc_1 complex, rather than the soluble cytochrome *c*-550 [18,19]. Even more surprisingly, *Rhodobacter capsulatus* mutants completely lacking their soluble cytochrome *c* are able to grow both by photosynthesis and by respiration [58]. This cytochrome usually donates electrons to the photosynthetic reaction centre and cytochrome oxidase. It appears likely that in its absence the bc_1 complex can react directly with the reaction centre [59], and possibly cytochrome oxidase as well. Interestingly, the closely-related *R. sphaeroides* is unable to grow in the absence of its soluble cytochrome

c. The bc_1 complexes in *capsulatus* and *sphaeroides* are functionally identical [60] and thus the species-specific interactions presumably reside in the reaction centre or the oxidase.

Although there is no evidence that mitochondrial cytochrome oxidase can function in the absence of a soluble cytochrome *c*, it is important to realize that the polarographic and spectrophotometric assays are *both* nevertheless 'unphysiological'. The ratio of cytochrome *c* to cytochrome aa_3 in mitochondria is frequently close to 1 : 1 [61] and never more than 2 : 1 [29], yet the ratios used in these assays are routinely as high as 1000 : 1. Attempts have been made to trap cytochrome *c* inside cytochrome oxidase proteoliposomes at low *c* : aa_3 ratios [62]. The resulting model system will more accurately mimic mitochondria, as the cytochrome *c* is now inside an enclosed space surrounded by high lipid and enzyme concentrations. Unfortunately, vesicle heterogeneity currently limits the use of this system for detailed kinetic studies [63].

However, despite the 'unphysiological' nature of the polarographic and spectrophotometric assays, their use in tandem to elucidate the steady-state kinetics of cytochrome oxidase is potentially very useful because of the valuable mechanistic information it can provide.

IV. Variation from simple Michaelis-Menten kinetics

Under low ionic strength conditions, both spectrophotometric [64] and polarographic [40] assays reveal a non-hyperbolic variation of reaction velocity with increasing cytochrome *c* concentration. As first-order kinetics are maintained in the spectrophotometric assay, Errede and Kamen [65] showed the data can be explained with the following phenomenological equation:

$$\text{velocity } (v) = k'[\text{oxidase}][\text{ferrocytochrome } c]$$

$$\text{where } k' = \frac{\alpha_1 + \alpha_2[\text{cyt.}c_{\text{total}}]}{1 + \beta_1[\text{cyt.}c_{\text{total}}] + \beta_2[\text{cyt.}c_{\text{total}}]^2}$$

$$\text{Thus } \frac{v}{[\text{oxidase}]} = \frac{\alpha_1[\text{cyt.}c^{2+}] + \alpha_2[\text{cyt.}c^{2+}][\text{cyt.}c_{\text{total}}]}{1 + \beta_1[\text{cyt.}c_{\text{total}}] + \beta_2[\text{cyt.}c_{\text{total}}]^2}$$

As shown by Myers and Palmer [66] when $[c^{3+}] = 0$ this equation reduces to:

$$\frac{v}{[\text{oxidase}]} = \frac{\alpha_1[\text{cyt.}c^{2+}] + \alpha_2[\text{cyt.}c^{2+}]^2}{1 + \beta_1[\text{cyt.}c^{2+}] + \beta_2[\text{cyt.}c^{2+}]^2}$$

An equation of this type can be rewritten as a sum of two Michaelis expressions [66]:

$$\frac{v}{[\text{oxidase}]} = \frac{V_{\text{max1}} \cdot [\text{S}]}{K_{\text{m1}} + [\text{S}]} + \frac{V_{\text{max2}} \cdot [\text{S}]}{K_{\text{m2}} + [\text{S}]}$$

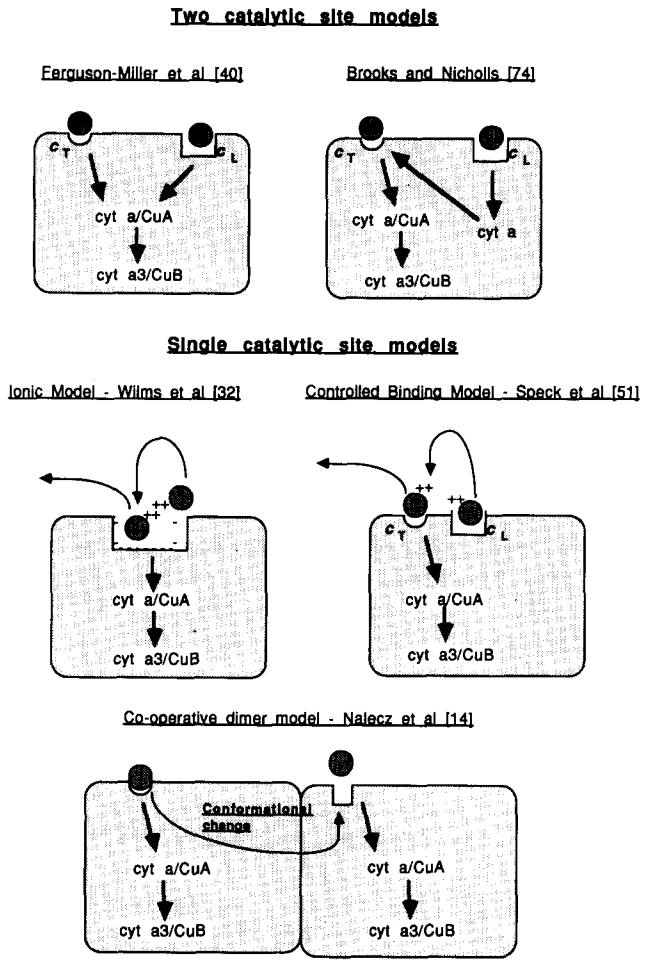

Fig. 2. Models of cytochrome oxidase steady state kinetics with two cytochrome *c* binding sites.

In the polarographic assay, cytochrome *c* is fully reduced in the steady state and thus $[c^{3+}] = 0$. The above equation, therefore, phenomenologically shows why both the initial rate of reduction of ferrocytochrome *c* (spectrophotometric assay) and the steady-state rate of consumption of oxygen (polarographic assay) do not have a simple hyperbolic dependence on the concentration of ferrocytochrome *c*. There have been many models proposed to explain this phenomenon, but they fall into two main schools of thought, depending on the presence or absence of two distinct cytochrome *c* binding sites.

IV-A. The two cytochrome c binding sites model (Fig. 2)

Before discussing the different models it is worth reviewing the evidence that supports the presence of two cytochrome *c* binding sites on cytochrome oxidase. The initial kinetic data of Ferguson-Miller et al. [40] compared the K_m values of the high- and low-affinity phases in the polarographic assay with direct cytochrome *c* binding studies (using gel filtration) and found a good correlation. The presence of low-affinity binding

sites has also been observed for a variety of primate and chemically modified cytochromes *c* [46,67]. Before accepting this as evidence in favour of the two sites model it is important to check whether the mechanism proposed in fact predicts that that the kinetic K_m should correspond to the K_D for substrate binding (there is frequently no such simple correlation [68]). For the polarographic assay it was shown [44] that, as TMPD reduces bound *c* faster than it dissociates, $T_N \propto$ [ES]. Under these conditions the Eadie-Hofstee plot becomes strictly analogous to a Scatchard plot and $K_m \cong K_D$. Even stronger evidence for a second cytochrome *c* active site comes from studies on the thermophilic bacterium, PS3, which contains one covalently bound cytochrome *c* [17]. TMPD alone can reduce the covalently bound *c* and produce high turnover. In this case the addition of external cytochrome *c* appears to catalyze electron transfer as if it were acting at a low-affinity site only – the kinetics are monophasic with a high K_m. This appears consistent with the presence of two binding sites. Analogous results to these have been obtained with another thermophilic cytochrome caa_3, *Thermus thermophilus* [69]. However, as direct binding studies of the proposed low-affinity form were not performed it remains possible that the exogenous *c* reduced the bound *c* directly, analogous to TMPD. This appears unlikely, however, as the effect of the added yeast cytochrome *c* decreased rapidly with ionic strength, just as in mitochondria. However, neither the oxidation of TMPD nor *T. thermophilus* cytochrome *c*-552 by PS3 were affected by ionic strength.

Recently Michel and Bosshard [70,71] have used the small perturbation in the spectrum of cytochrome *c* upon binding to the oxidase as a means of monitoring cytochrome *c* binding photometrically. They then compared the degree of binding to the observed spectrophotometric rate of cytochrome *c* oxidation [71]. Under conditions where the high-affinity K_m for the bovine enzyme was 0.1 μM and the low-affinity K_m was 8 μM, only one dissociation constant for cytochrome *c* could be observed (0.29 μM). However, the binding assay would not have detected a second weaker cytochrome *c* binding site with a dissociation constant of up to 20 μM. In contrast, ultracentrifugation measurements of the binding of the second molecule of cytochrome *c* are in good agreement with the low-affinity spectrophotometric K_m [72], suggesting that binding at the low-affinity binding site may not affect the cytochrome *c* spectrum and thus not be directly monitored by the photometric binding assay.

Michel and Bosshard have also claimed that the difference between the high-affinity K_m measured spectrophotometrically and the K_d measured by their binding technique for a variety of cytochromes *c* [71,73] is evidence against the two cytochrome *c* binding model. However, the equivalence of the high-affinity K_m with the high-affinity K_d, whilst a consequence of the Minnaert mechanism (see section II), is not essential to the argument that the two K_m values result from two separate cytochrome *c* molecules binding to the oxidase. Furthermore, for the horse cytochrome *c*/bovine cytochrome oxidase system there is a good correlation of the spectrophotometric high-affinity K_m and the K_d when the latter is measured by gel filtration [72] or the photometric binding assay [71] (the large differences noted by Michel et al. in [73] come from an incorrect comparison of kinetic and binding data measured under different conditions in Ref. 71). This suggests that the deviations from the Minnaert mechanism observed by Michel and Bosshard may only occur when the cytochrome *c* molecule employed in the assay is significantly different from the physiological cytochrome.

IV-A1. Two catalytic site models

Given that there are two cytochrome *c* binding sites, how does the binding of the second cytochrome *c* molecule increase the rate of electron transfer? The simplest explanation is that the second cytochrome *c* molecule is catalytically active. This was first proposed by Nicholls [39]. In this model, the cytochrome *c* bound at the low-affinity site (c_L) can transfer electrons to the cytochrome *c* at the high-affinity site (c_T), either directly or via cytochrome *a* or Cu_A [74]. This can increase the reaction rate in a manner analogous to TMPD in the polarographic assay – by allowing another turnover in the absence of product dissociation. In the polarographic assay it must be presumed that reduction of c_T by c_L is faster than that by TMPD, or no second phase would be seen. However, unlike the two catalytic sites model proposed by Ferguson-Miller et al. [40], the c_L site is not presumed to be fully functional alone as it can only reduce cytochrome *a*, whereas c_T can reduce *a* and a_3 [55].

Perhaps the strongest evidence in favour of these models is that the presence of porphyrin cytochrome *c* (lacking an iron centre) at the c_T site has been shown to have no effect on the rate of the pre-steady-state reduction of cytochrome oxidase by ferrocytochrome *c* at the c_L site [75]. Thus, it appears that the c_L site can reduce cytochrome *a* directly. Presuming that in the mitochondrion the c_T site is always occupied [55], the c_L site has been postulated [74] to be the mediator for intermembrane electron exchanges, e.g., from NADH cytochrome b_5 reductase [76]. Clearly, to be involved in such a function the c_L site must be catalytically active.

The two catalytic site mechanism has come under some criticism. There have been suggestions that in mitochondria there are more than two phases to the Eadie-Hofstee plots in the spectrophotometric assays [77], which would mitigate against a simple two catalytic site model. However, this finding has been disputed [33].

IV-A2. One catalytic site models

The essence of all single catalytic site models is that the binding of a molecule at the second cytochrome *c* binding site influences the reaction at the first site. The 'dependent site' mechanism proposed by Errede and co-workers [64,65] says nothing about what this interaction might be. In what they term the 'controlled binding hypothesis' [42], Speck et al. [51] presumed that this interaction involved an effect on the binding of the cytochrome *c* at the tight site (possibly by electrostatic repulsion). Thus, if product dissociation is rate limiting the binding of the second cytochrome *c* molecule will increase the rate of reaction by increasing product release and the Eadie-Hofstee plots will be curved.

Binding studies have suggested that the second molecule of cytochrome *c* binds to cardiolipin or other acidic phospholipids associated with the oxidase complex [78,79]. Indeed the kinetics at the low -affinity site are specifically affected by alterations in the lipid composition of detergent-solubilized [79] proteoliposomal [77] and mitochondrial [80] cytochrome oxidase. However, these kinetic differences may be secondary to lipid effects on enzyme dimerization [81]. Furthermore, recent studies on the delipidated enzyme [82] have shown that a low-affinity reaction is observable in the presence of dimyristol phosphatidylcholine alone, suggesting that either the low-affinity binding site is not composed of acidi phospholipids or that two cytochrome *c* binding sites are not necessary to observe biphasic kinetics.

As an alternative to a 'controlled binding' hypothesis it is also possible for biphasicity to be explained by cooperativity between two equivalent sites on the oxidase dimer. Indeed there is some evidence that the monomer has monophasic kinetics. *P. denitrificans* cytochrome aa_3 is monomeric and shows monophasic kinetics [83], and removing subunit III from the bovine heart enzyme has been reported to increase the number of monomers and linearize the Eadie-Hofstee plots [14]. However, other workers have observed biphasic kinetics in largely monomeric preparations [13,16,71,84,85]. The concept of strong interactions between oxidase dimers is though the easiest way to explain the finding of Bisson et al. [78] that one molecule of arylazidocytochrome *c* per dimer is sufficient to totally inhibit electron transfer. The dimeric model would be clearly distinct from the previous ones discussed in that, despite involving interaction between separate cytochrome *c* binding sites, it would not require a stoichiometry of greater than one *c* per aa_3.

IV-A3. Ionic interaction model

An alternative theory has been put forward by Wilms et al. [32] that is half-way between a one and a two binding site model. They suggest that there is only one binding site for cytochrome *c*, but that it is an ill-defined negative region of the oxidase that can bind two

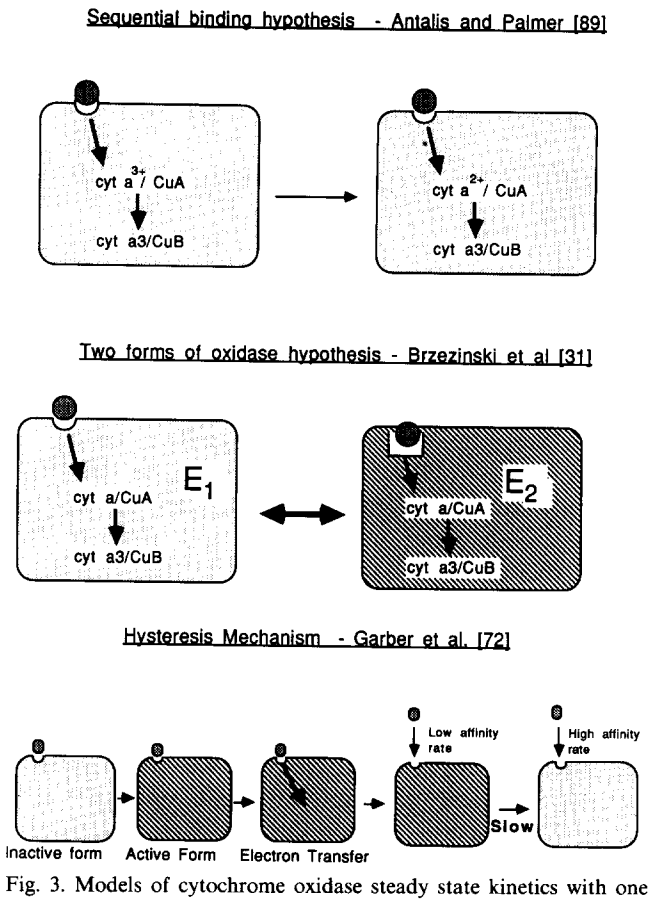

Fig. 3. Models of cytochrome oxidase steady state kinetics with one cytochrome *c* binding site.

cytochrome *c* molecules simultaneously. The binding of the second cytochrome *c* results in an electrostatic repulsion between the two positively charged molecules. Thus cytochrome *c* dissociation is favoured and the reaction rate increases as described above. In favour of this model are data suggesting that cytochrome *c* molecules can repulse each other electrostatically in solution [86,87], but it is inconsistent with a wealth of data suggesting that the high-affinity site is distinct and has a 1 : 1 stoichiometry [51]. However, both these facts are consistent with the model of Speck et al. discussed earlier, and the single binding site nature of this hypothesis appears to have been abandoned [33,88].

IV-B. The one cytochrome c binding site model (Fig. 3)

IV-B1. Sequential binding hypothesis

Antalis and Palmer [89] were the first to suggest a model of oxidase kinetics that did not include two cytochrome *c* binding sites. Instead, two cytochrome *c* molecules reacted sequentially at the same site, with identical association and dissociation constants. This leads to rate equations containing higher degree terms of substrate concentration and can thus explain the kinetic data. However, the pre-steady-state data reported to support this model do not correlate well with

the steady-state parameters [46,77,90], and recently Myers and Palmer [66] have shown that such sequential binding models fail to predict non-hyperbolic behaviour when $[c^{3+}] = 0$, i.e., in the polarographic assay.

IV-B2. Two forms of oxidase hypothesis (conformational transition)

A radicaly different interpretation of the non-hyperbolic kinetics has recently been proposed by Malmström and co-workers [31,91,92]. The essential argument is that, rather than assuming there are two separate binding sites on the same oxidase molecule, they assume there are two separate forms of the enzyme which react with the substrate differently (for a similar argument in relation to the ATP synthetase kinetics see Ref. 93). Thus only one cytochrome *c* molecule ever binds to the enzyme at one time, but there are two forms of the enzyme that it can bind to with different affinity. Therefore, it can clearly be seen that this will lead to biphasic kinetics. The model has been refined and is in good agreement with the experimental data [94,95].

Why presume there are two forms of the enzyme, however? Malmström argues, like Wikström et al. [12], that redox-linked proton pumps must have two oxidized forms, one to pick up protons from the input face and the other to releaase them on the output face. However, unlike Wikström, Malmström proposes that both form react profitably with substrate. If the input and output forms have different substrate affinities this can lead to non-hyperbolic kinetics. The differing substrate affinities are assumed to arise from the different potential of cytochrome *a* in the two forms (E_1 and E_2) due to the redox interaction with cytochrome a_3 [12,96] and/or the pH dependence of the midpoint potential [12]. The model also depends on the two-electron gating hypothesis, originally proposed by Antonini et al. [97] which suggests that both Cu_A and cytochrome *a* must be reduced before electron tranfer to the Cu_B/cytochrome a_3 centre can occur. Although both Malmström's interpretation of the redox interaction and his electron gating hypothesis are not universally accepted (see Nicholls and Wrigglesworth [98] and Wrigglesworth et al. [99] for recent alternative theories) it is clear that such a hypothesis could explain the biphasic kinetics.

Perhaps a more fruitful critique of this model arises from its strong linking of non-hyperbolic kinetics with the proton pumping capability of the enzyme. The model has great difficulty in explaining the presence of homologous eukaryotic and prokaryotic enzymes that are known to pump protons, but have monophasic kinetics such as yeast [41], PS3 [17] and *P. denitrificans* [83]. Can the proton pumping mechanism be radically different in these cases? Are the redox interactions between cytochrome *a* and cytochrome a_3 different? Or have the correct conditions to observe the biphasic kinetics in these organisms not been found yet? Recent data showing that both yeast [80] and *Paracoccus* [71] can exhibit biphasic kinetics suggest that at least for these organisms the latter explanation may be correct.

Some of the criticisms Malmström makes of the single catalytic site hypotheses are ill-founded. He makes the useful point that talk of a single rate-limiting step in such a complicated enzyme cycle is naive – as it can be shown that even individual steps with high rate constants can limit the overall flux to some extent if there are enough of them [91]. Similar ideas have been developed extensively in analyzing fluxes in whole metabolic pathways [100,101]. Thus in Malmström's model, the best fit is obtained when the rate constants for product dissociation and intramolecular electron transfer are comparable [31], and changing either can therefore affect the rate of electron transfer. However, it does *not* follow from this, as Malmström suggests, that an explanation of the biphasic kinetics based on an increase in the rate of product dissociation is incorrect. As long as the rates of product dissociation and internal electron transfer are comparable, then increasing the former by binding a second molecule of cytochrome *c* will increase the flux through the whole enzyme – and lead to biphasic kinetics. As under most conditions the V_{max} of the high-affinity reaction is within an order of magnitude of that of the low-affinity reaction [74,88] this does not appear too unlikely. As mentioned previously, it does not follow from the Minnaert mechanism that the rate of internal electron transfer cannot be partially rate-limiting, nor is such a requirement essential to the two binding site hypotheses. In fact, as the binding of the second molecule of cytochrome *c* is suggested by the controlled binding hypothesis to increase the rate of product dissociation, at saturating cytochrome *c* concentrations product dissociation is not expected to be rate-limiting, in agreement with Sarti et al. [30]. It is also one of the strengths and weaknesses of the dependent site and controlled binding hypotheses that they can be elaborated to fit different rate-limiting steps, e.g., by invoking a conformational change dependent on the binding of the second molecule of cytochrome *c* that increases the rate of internal electron transfer.

Finally it has been recently suggested by Myers and Palmer [66] that in its present form the Malmström model is unable to fully explain cytochrome oxidase kinetics as it does not lead to first order cytochrome *c* oxidation kinetics in the spectrophotometric assay.

However, despite these criticisms the Malmström model has the advantage that it attempts to explain cytochrome oxidase kinetics in terms of many reaction intermediates. The attempt to use kinetics to 'get inside' the reaction mechanisms is surely the correct approach, and will lead to further useful experiments and discussion (see Bickar et al. [102] and Myers and Palmer [66] is this light also).

Recently, two other groups have followed Malm-

ström's lead in suggesting that different conformational forms of the oxidase may be important in explaining the biphasic kinetics. Bolgiano et al. [26] have suggested that cytochrome *c* binding leads to a conformational change that increases the rate of internal electron transfer. It is important to remember that a substrate-induced conformational change will not lead to biphasic Eadie-Hofstee plots of the type exhibited by cytochrome oxidase unless there are two forms of the enzyme with different affinities for cytochrome *c*. To illustrate this two simple schemes can be studied:
the substrate-induced conformational change is necessary for electron transfer:

$$E + S \rightleftharpoons ES \longrightarrow ES' \longrightarrow E + P$$

Scheme 1

the conformational change produces an increase in the rate of electron transfer:

$$\begin{array}{ccccccc} E + S & \rightleftharpoons & ES & \longrightarrow & ES' & \longrightarrow & E + P \\ & & \downarrow & & & & \\ & & E + P & & & & \end{array}$$

Scheme 2

However, both these mechanisms [103] produce rate equations of the form:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

and therefore cannnot account for non-hyperbolic behaviour.

Stating that product dissociation is not rate-limiting in the polarographic assay and that no second molecule of cytochrome *c* is bound during the biphasic switch in the assay, Margoliash's group [42,72] have rejected their 'controlled binding' hypothesis as an explanation for the polarographic kinetics. Instead they have introduced a 'hypsteresis mechanism', whereby the oxidase needs to enter an activated state after binding ferrocytochrome *c* before it can accept electrons. After product dissociation, the enzyme can relax to the slow reacting form. However, there is a chance that the enzyme can react with a cytochrome *c* molecule before it has relaxed to the slow reacting form – a chance dependent on the concentration of cytochrome *c* present. Therefore, at high cytochrome *c* concentrations, the reaction will deviate from Michaelis-Menten kinetics. This model differs from the Malmström model in that the biphasic kinetics is not directly linked to enzyme function. This suggests that the low-affinity reaction may have evolved specifically to control enzyme turnover [72], rather than as a consequence of the proton translocating mechanism.

This hysteresis mechanism is in fact identical to that proposed by Rabin as a kinetic model for the behaviour of co-operative enzymes [104]. It is illustrated below:

$$\begin{array}{ccccc} S + E & \rightleftharpoons & ES & & \\ \uparrow & & \downarrow & & \\ S + E' & \rightleftharpoons & E'S & \longrightarrow & E' + P \end{array}$$

Scheme 3

If the step $E'S \rightarrow E' + P$ is fast compared to $E' \rightarrow E$ and $ES \rightarrow E'S$, then at high [S] the enzyme will exist mostly as E'S. The reaction will therefore be rapid as it bypasses the slow steps ($E' \rightarrow E$ and $ES \rightarrow E'S$). However, at low [S] the enzyme will have time to relax from $E' \rightarrow E$ and thus the reaction will proceed via the slow step $ES \rightarrow E'S$. Therefore, there will be a lag phase in the plot of v vs. [S], which will appear sigmoidal (co-operative behaviour). However, if $E'S \rightarrow E' + P$ is not fast compared to $E' \rightarrow E$ and $ES \rightarrow E'S$, and the substrate affinities are significantly different for E and E', then this model will show apparent negative cooperativity, with a low K_m, low V_{max} phase and a high K_m, high V_{max} phase. Thus, it can display the biphasic Eadie-Hofstee plots associated with cytochrome oxidase activity. Indeed, the steady-state rate equation for the Rabin mechanism can be converted [103] into the form:

$$v = \frac{V_{max1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{max2} \cdot [S]}{K_{m2} + [S]}$$

Therefore, a substrate-induced conformational change can explain the biphasic kinetics of cytochrome oxidase activity if, and only if, ferrocytochrome *c* can bind to both enzyme conformations.

It is instructive to compare the Rabin mechanism with the conformational transition mechanism discussed earlier. Illustrating the minimal Malmström mechanism [91] in a similar form to the Rabin model we get:

$$\begin{array}{ccccccccc} S + E_1 & \overset{fast}{\rightleftharpoons} & E_1S & \longrightarrow & E_1' + P & \longrightarrow & E_2'' & \longrightarrow & E_2 \\ \updownarrow & & & & \updownarrow & & & & \\ S + E_2 & \overset{slow}{\rightleftharpoons} & E_2S & \longrightarrow & E_2' + P & & & & \end{array}$$

Scheme 4

Again the ratio of the fully oxidized low-affinity form E_2 to the fully oxidized high-affinity form E_1 will increase as the substrate concentration is increased. However, because of the fact that E_2 converts back to E_1' before completing the reaction cycle and there are reversible reactions between the different forms of E_1 and E_2, the overall E_1/E_2 ratio is hardly affected by changes in [S].

V. Ionic strength effects

It has long been known that at a given cytochrome *c* concentration a plot of ionic strength against steady-state activity of cytochrome oxidase at pH 7–7.8 rises to an optimum and then declines to close to zero. This occurs both in the spectrophotometric [32,105,106] and polarographic [107] assays. However, when separate plots of V_{max}, V_{max}/K_m and K_m are plotted they show no such maximum [32,108]. This paradox has been shown to be an artifact due to the fact that a combination of low- and high-affinity reactions are being observed [88]. What then are the effects of ionic strength on the high- and low-affinity reactions? There has been some dispute about this, but recently a consensus has emerged. The K_m and V_{max} of the high-affinity reaction both increase rapidly with increasing ionic strength [74,88]. Although the K_m of the low-affinity reaction increases rapidly with increasing ionic strength, the V_{max} increases only slowly, if at all [74,88,90]. The effect of these increases in V_{max} *and* K_m are to give an apparent maximum in plots at a single cytochrome *c* concentration. Upon initially increasing ionic strength the increase in V_{max} at the high-affinity site is most marked. Then the increase in both high- and low-affinity K_m values overshadow the V_{max} increases, resulting in a decrease in measured activity.

How does ionic strength affect the kinetics of cytochrome oxidase? On the basis of small spectral changes in the bovine heart enzyme, Kadenbach and co-workers have recently suggested that the principal effect of ionic strength is mediated via anion-specific binding to the enzyme – resulting in conformational changes that affect enzyme activity [109]. However, a re-plot of their data allowing for the different ionic strengths employed showed very little difference between anions [107]. This is in agreement with the findings of Wilms et al. who showed [32] that replacing phosphate with chloride at the same final ionic strength had no effect on activity, and that there was no effect of ionic strength on the activation enthalpy of the pre-steady state reactions of the enzyme [110]. Thus it seems that the primary effect of increasing ionic strength is an anion-independent effect on an electrostatic interaction.

At high ionic strengths Eadie-Hofstee plots become monophasic. Both Brooks and Nicholls [74] and Sinjorgo et al. [88] agree that this is due to the increase in the K_m of the low-affinity reaction being so large that its effect on turnover (via whatever means) is no longer observed. Thus at high ionic strength only the high-affinity reaction is measurable. However, because these authors have different views as to what is occurring at the loose site, they have different explanations for the effect of ionic strength. Brooks and Nicholls [74] suggest that as the two sites do not directly interact, high ionic strength must prevent effective binding at the loose site, presumably due to electrostatic interactions. According to Singjorgo et al. [88], however, the effect exerted by the loose site on turnover is via an ionic interaction. Therefore, even if the cytochrome *c* molecule could bind at the loose site at high ionic strength, it could not affect turnover – as it essentially acts via the same mechanism as ionic strength. Both groups agree that the principle effect of ionic strength at the tight site must be to increase K_m by decreasing the rate of substrate binding and to increasing V_{max} by increasing the rate of product dissociation. As expected, if its primary effect is via increasing the product dissociation rate, the effect of ionic strength on V_{max} is more pronounced in the spectrophotometric assay [74] where this is 'rate limiting'. The fact that similar, albeit smaller, effects are seen in the polarographic assay, however, is a further caveat against presuming one step in a complex reaction is always rate limiting and solely controls the flux (cf. section IV-B2.).

It follows from the above that at saturating cytochrome *c* concentrations under low ionic strength conditions two cytochrome *c* molecules are bound, whereas at high ionic strengths only the high-affinity site is occupied. The relative equivalence of the maximal rate of enzyme turnover at low and high ionic strengths has thus been taken by Brooks and Nicholls [74] as evidence against two independent catalytic sites on the oxidase, although still consistent with models where electrons are transferred from the c_L site to the c_T site.

The single binding site model of Malmström must clearly have a different explanation for the effect of ionic strength on the reaction. The assumption made here [91] is that the form of the enzyme that reacts with high affinity to cytochrome *c* (E_1), does so in an ionic strength-dependent manner, whereas the form that reacts with lower affinity (E_2) is less ionic strength-dependent. Therefore at high ionic strength, even though a significant population of enzyme molecules exist in the E_1 form, their rate of reaction with cytochrome *c* is now so low that essentially all cytochrome *c* oxidation occurs via the E_2 form.

Finally, it is also possible that increasing the ionic strength may influence the oxidase kinetics by affecting the monomer/dimer equilibrium, as suggested by Azzi and co-workers [106]. However, given the ability of many monomeric oxidase preparations to exhibit biphasic kinetics cf. section IV-A2), this is unlikely to explain the fundamental switch from biphasic to monophasic kinetics following an increase in ionic strength.

VI. pH effects

The effects of pH on the steady state kinetics of cytochrome *c* oxidation by cytochrome oxidase have been extensively studied for several reasons: (i) Protons are consumed in the oxygen reduction reaction and are

hence a substrate for the enzyme. (ii) The proton translocation activity of the enzyme must involve the binding and release of protons by the enzyme, both steps that could affect the kinetics of cytochrome *c* oxidation. (iii) The establishment of a membrane potential and/or a pH gradient across a membrane (mitochondrial or proteoliposomal) can inhibit cytochrome oxidase activity. An understanding of the pH dependence of cytochrome *c* oxidation may illuminate the mechanism of this respiratory control. The pH dependence of oxidase turnover can be studied in the presence or absence of a proton motive force.

VI-A. pH dependence of uncoupled enzyme turnover

Early studies revealed an optimal pH for cytochrome oxidase turnover [28,111–116]. However, the position of this optimum varied depending on the experimental conditions, and it is now agreed that maximum enzyme turnover increases with decreasing pH up to the experimental limits (pH 5) [88,90,117,118]. Several technical difficulties explain the previous problems in observing this:

(i) If turnover is measured at a single cytochrome *c* concentration an increase in K_m can decrease enzyme turnover, despite an increase in V_{max} (similar anomalies occur when ionic strength is varied, cf. section V). This can explain the existence of an apparent pH optimum under conditions of high ionic strength where the Lineweaver-Burk plots of cytochrome *c* oxidation kinetics at different pH's intersect [118] but not at low ionic strengths where such intersections occur only at very low cytochrome *c* concentrations [88].

(ii) As ionic strength has a dramatic effect on the kinetic parameters (see previous section), varying the buffer concentration without allowing and compensating for the change in ionic strength will result in a combination of pH and ionic strength effects being observed.

(iii) Cytochrome oxidase preparations are unstable at low pH values (pH < 5). Such irreversible denaturation will clearly result in an apparent pH optimum for turnover that does not represent a true kinetic parameter. Some detergent-solubilized enzyme preparations are more acid-resistant than others [117]. Incorporating the enzyme into proteoliposomes can also markedly increase the enzyme resistance to such acid denaturation [119]. Unfortunately, at low ionic strengths these vesicles can aggregate at lower pH values, decreasing oxidase activity [118]. Such effects are especially important when Keilin-Hartree submitochondrial particles are used, where they cause the V_{max} to *increase* with increasing pH in both the polarographic [40] and spectrophotometric assays [116]. At high ionic strengths, however, the proteoliposomal and soluble oxidase show similar pH effects on cytochrome *c* oxidation kinetics (compare Wilms et al. [117] with Thörnström et al. [90] and Gregory and Ferguson-Miller [118]).

(iv) pH dependent non-enzymatic reactions can affect the rate of cytochrome *c* oxidation in the polarographic assay. In the absence of TMPD, ascorbate reduction of cytochrome *c* is inhibited by a decrease in pH [120]. The addition of TMPD can overcome this problem, ensuring complete reduction of cytochrome *c* in the steady-state from pH 5 to pH 8.5 [118]. However, under low ionic strength conditions, TMPD can directly reduce enzyme-bound ferricytochrome *c* (cf. earlier). This reaction is increased at high pH [121]. Therefore, in order to solely study the effect of pH on the *enzymatic* reactions, the polarographic assay must be carried out at high ionic strengths where product dissociation is not rate-limiting.

As was shown to be the case with the effect of ionic strength on oxidase turnover, awareness of such kinetic anomalies has recently resulted in a consensus emerging as to the effects of pH on the cytochrome *c* oxidation kinetics, if not on the mechanistic significance underlying such effects.

For the reasons stated above (iv), the effect of pH on enzyme turnover at low ionic strength ($I = 25$ mM) has been studied using the spectrophotometric, rather than the polarographic, assay. Sinjorgo et al. [88] found that both the low- and high-affinity V_{max} increased continuously as the pH was decreased from pH 8.6 to 5.4. A similar increase was seen in the high-affinity K_m. However, the low-affinity K_m was pH-independent. The combined effect of these changes is that Eadie-Hofstee plots of cytochrome *c* oxidation becomes increasingly monophasic as the pH is lowered [71,88].

At high ionic strengths ($I = 0.5$ M) the spectrophotometric assay shows a continuous increase in V_{max} and K_m as the pH is lowered from 8.5 to 5.0 [117], with no effect on V_{max}/K_m [90,95]. Under conditions of high ionic strength the polarographic and spectrophotometric assays give similar values for V_{max} and K_m [118] at pH 7.5 and 8.5. In agreement with the spectrophotometric assay, when assayed polarographically both V_{max} and K_m increase continuously as the pH is decreased from pH 8.5 to 5.0 [118]. However, whilst V_{max}/K_m is constant from pH 8.5 to 6.0 there is a significant decrease in V_{max}/K_m as the pH is further lowered to 5.0. It is not clear what is the reason for the different effects of low pH on the ratio of V_{max}/K_m in the two assays. However, as the K_m for cytochrome *c* is already high at the high ionic strengths used, and it is further raised due to the low pH, these data points are most liable to experimental error as it is not possible to measure enzyme turnover at substrate concentrations much higher than the K_m. This is especially a problem in the spectrophotometric assay where the high absorbance at the high cytochrome *c* concentrations can interfere with the assay [118]. Thus the differing effects on V_{max}/K_m when

measured spectrophotometrically and polarographically may be due to technical difficulties, rather than differences in the assay systems themselves.

The above consensus has emerged from studies on the oxidation of horse heart cytochrome *c* by both the bovine heart and rat liver enzymes. However, there is evidence that similar kinetics are shown by prokaryotic cytochrome oxidases. Both *P. denitrificans* [122] and *Bacillus subtilis* [123] show increased turnover at decreased pH values, although detailed kinetic studies have not been undertaken.

VI-B. Mechanism of pH effects on uncoupled turnover

The effects of pH on the maximum rate (V_{max}) of cytochrome *c* oxidation by cytochrome oxidase can be best modelled by the combined effects of three separate protonation/deprotonation steps, each of which increases enzyme turnover [90,117,118]. These three independent studies yielded similar values for the three pK_a values involved (4.5–5.0, 6.2–6.8 and 7.8–8.0). In order to explain the pH dependence of the reduction potential of haem *a*, Wikström et al. [12] required the presence of three acid-base groups, each with two pK_a values (4.5–6.5; 4.5–6.5; and 6.5–8.0). However, as pointed out by Thörnström et al. [90], it is important to remember that the pK_a values derived from kinetic measurements do not represent thermodynamic constants and thus the above similarity may be coincidental. Indeed, with such a complex reaction mechanism the pK_a values determined from the pH dependence of the kinetic constants in cytochrome oxidase are unlikely to be directly related to pK_a values from groups in the enzyme active site [90].

In a Michaelis-Menten mechanism the ratio V_{max}/K_m is the initial tangent to a plot of v against [S] and thus represents the rate constant for the second-order reaction of enzyme and substrate in the absence of the formation of an enzyme substrate complex [22]: $E + S \rightarrow E + P$. The lack of a significant pH dependence of the high-affinity V_{max}/K_m, despite the pH dependence of the two parameters, suggests that the rate of enzyme/substrate association is not a limiting factor in the rate of cytochrome *c* oxidation as the pH is lowered from 8.5–6.0 [90]. Sinjorgo et al. [88] have suggested, however, that the pH dependence of the high-affinity K_m and that of the high-affinity V_{max} can be explained similarly, if it is assumed that the rate of product *dissociation* is limiting as the pH is changed. Therefore, the protonation of a residue on subunit II (the cytochrome *c* binding subunit) could affect both the affinity for the substrate and the maximal turnover rate. However, kinetic modelling [94,118] suggests that at least under the high ionic strength conditions used by Thörnström et al. [94] and Gregory and Ferguson-Miller [118], the rate of product dissociation becomes faster than k_{cat} as the pH is lowered, suggesting that pH effects on enzyme turnover are not mediated via effects on the rate of ferricytochrome *c* dissociation from the oxidase.

If the pH effects are not related to substrate binding or product dissociation, then reactions in the catalytic cycle must be pH dependent [71,90,117]. If this is the case there should be a kinetic isotope effect on enzyme turnover. An inhibitory effect of D_2O on enzyme turnover has been observed for the solubilized [124] and vesicular [90] enzyme, although there was no effect on the pre-steady-state reactions [110]. This discrepancy arises from whether the results in D_2O are displaced by 0.54 pH units when compared to the results in H_2O (presuming $pK_a^D = pK_a^H + 0.54$ [125]). If this is done, then a solvent isotope effect of about 2 is observed, although there is no effect on the ratio V_{max}/K_m [90].

What are the individual reaction steps that might be pH dependent? Clearly the rate of the association of the scalar protons consumed by the oxidase in the reduction of water must be affected by the pH [117]. However, proton equilibria with side chains in highly buffered solutions are generally very rapid [90] and under these conditions the availability of protons for the reduction of oxygen intermediates does not appear to be rate-limiting [126]. It is possible that there is direct proton/hydroxyl binding to the prosthetic groups of the enzyme. Wilms et al. [110,117] suggest that the binding of OH^- as the sixth ligand of haem a_3 as proposed by Lanne et al. [104] may inhibit turnover. It has also been proposed that at low pH a hydrogen bond between the oxidase protein and the formyl group of the haem a^{2+} porphoryin ring is broken, although this does not appear to correlate with the increased enzymatic turnover at low pH [118].

The proton translocating activity of cytochrome oxidase must involve protonation and deprotonation reactions. The model of Malmström and co-workers mentioned earlier [91] proposed that there are two forms of the oxidase – E_1 and E_2. Recently it has been suggested [128] that it is the conformational transition between these two forms, rather than the internal electron transfer reaction, that is rate-limiting for the oxidase reaction. If the binding of a proton to the input site of the proton pump (E_1) occurred at a low pK_a, then this rate would limit the $E_1 \rightarrow E_2$ transition. Such an explanation gives a reasonable fit to the data describing the pH dependence of enzyme turnover [95]. A similar mechanism has been proposed by Michel and Bosshard [71]. However, showing that a kinetic mechanism is consistent with the data does not guarantee that it is correct. Indeed, a full explanation of the pH effects on the steady-state oxidase reaction is likely to be far more complex, given the need for at least three separate pK_a values to explain pH effects on V_{max}, as well as those that may be involved in the effects on K_m.

VI-C. pH dependence of turnover in the presence of a proton motive force

The pH effects described above can potentially provide important information about the mechanism of cytochrome oxidase turnover. However, in vivo, cytochrome oxidase turnover generates a proton motive force across the inner mitochondrial membrane consisting of a membrane potential (negative inside) [129–131] and a pH gradient (alkaline inside) [130]. Both these gradients can inhibit cytochrome oxidase turnover [132,133]. Thus a study of the pH dependence of enzyme turnover in the presence of a proton motive force may shed some light on the mechanism of this respiratory control.

Both polarographic [134] and spectrophotometric assays [135] show an approximately linear increase in coupled proteoliposomal cytochrome oxidase turnover as the initial pH (inside and outside the vesicles) is lowered from 8.0–5.0. However, the turnover in the presence of uncouplers (and the consequent absence of a $\Delta\mu H^+$ across the vesicle) rises to a plateau with decreasing pH [134,135]. The combined effect of this is a decrease in the ratio of the uncoupled to coupled turnover (respiratory control ratio or RCR) with decreasing pH. Although similar results are obtained with *Bacillus subtilis* [123] cytochrome oxidase, the uncoupled turnover drops more dramatically at higher pH values, resulting in an optimum value of the respiratory control ratio at about pH 7. Such an optimum can also be seen with bovine heart cytochrome oxidase under some conditions (Cooper, unpublished observations). Discrepancies such as these are not surprising as the experiments have been performed at a single cytochrome *c* concentration under different ionic strength conditions. A full analysis of the differences in the pH dependence of the coupled and uncoupled turnover will require experiments at a variety of ionic strengths and cytochrome *c* concentrations, as well as measurements of the proton motive force itself.

However, this preliminary data has lead to some tentative conclusions. Maison-Peteri and Malmström [135] have suggested that the reduction in respiratory control ratio (RCR) at low (non physiological) pH values is due to 'slip' in the enzyme, i.e., electron transfer in the absence of proton translocation. Indeed there is evidence that the H^+/e^- ratio for proton translocation declines at decreasing pH [134]. However, as the steady-state pH gradient and membrane potentials were not measured in these experimens, there is no direct evidence that such a slip is the cause of the decrease in RCR. Indeed, the removal of subunit III which significantly decreases the H^+/e^- ratio, can result in a significant *increase* in the respiratory control ratio [119]. In addition the use of low concentrations of *N,N'*-dicyclohexylcarbodiimide (DCCD) to decrease the H^+/e^- ratio has little effect on the RCR [136] although there is a small decrease in the steady state pH gradient [137] and membrane potential [136].

How might the control of enzyme turnover by a proton motive force across the proteoliposomal (and mitochondrial) membrane be mediated? As mentioned by Brand and Murphy [101] it is important to distinguish ΔpH effects from those exerted separately by pH_i or pH_o. In the absence of a membrane potential (+valinomycin) the maximum pH gradient observed in cytochrome oxidase proteoliposomes is 0.5–0.6 [137–139]. Is it possible to explain the increase in respiration when this gradient is collapsed solely by the pH effects on turnover observed in the uncoupled state [135]? Under conditions when low respiratory control ratios are observed this may be possible [135,140,141]. However, collapsing the ΔpH for proteoliposomes respiring in a buffered pH 7.5 medium (with internal pH therefore of ca. 8) can under certain conditions increase the respiration rate from 6 [142] to 15-fold [143] – yet, under similar conditions the rate of uncoupled turnover only doubles from pH 8.0 to 7.0 [143]. Apparently, the enzyme can sense the thermdynamic ΔpH across the membrane [144] and the effects of this gradient on turnover cannot therefore be simply explained by examining the pH dependence of the uncoupled turnover. Alternatively, it is possible that the steady-state ΔpH is dramatically underestimated in these systems due to vesicle heterogeneity (as shown with bacteriorhodopsin proteoliposomes [145]), although such effects do not appear to be large enough to explain these discrepancies [142].

The mechanism of this control is controversial. Studies on the redox state of haem *a* and a_3 in coupled and uncoupled cytochrome oxidase proteoliposomes [143,144,146] have suggested that the rate of electron transfer from cytochrome *c* to haem *a* is controlled by $\Delta\Psi$, whereas electron transfer from haem *a* to haem a_3 is predominantly controlled by the pH gradient. However, if the cytochrome *c* concentration is varied over a wide range a more complex pattern emerges [147]. Furthermore, a purely thermodynamic effect of the gradients on the redox centres has difficulty in explaining the residual enzyme turnover seen at high values of $\Delta\mu H^+$ [147]. In order to accommodate these findings Nicholls [147] has proposed an alternative, allosteric model for oxidase control where ΔpH converts the enzyme into a form with a slower rate of electron transfer from haem *a* to a_3 (a similar model explaining the effects of $\Delta\Psi$ on turnover has been previously proposed by Brunori et al. [148]). However, the results [147] are also consistent with a model where $\Delta\mu H^+$ can control the rate of turnover by increasing the rate of reversed electron transfer between the a_3/Cu_B centre and cytochrome *a* [147,149].

VII. Conclusions

The steady-state rate of cytochrome *c* oxidation by cytochrome oxidase is affected by several factors. Any correct kinetic mechanism must account for: (i) The first-order time-course of cytochrome *c* oxidation observed in the spectrophotometric assay. (ii) The non-hyperbolic variation of turnover with increasing cytochrome *c* oxidation observed in both the polarographic and spectrophotometric assay at low ionic strengths. (iii) The fact that both product dissociation and internal electron transfer rates can limit the flux under certain conditions. (iv) The increase in both the low-affinity and the high-affinity K_m values for cytochrome *c* with increasing ionic strength. (v) The increase in the high- but not the low-affinity V_{max} with increasing ionic strength. (vi) The increase in the high-affinity K_m with decreasing pH, whereas the low-affinity K_m is pH independent. (vii) The increase in both the low- and high-affinity V_{max} values with decreasing pH.

A requirement for an enzyme-substrate complex appears the most satisfactory explanation of the oxidase steady-state kinetics at present (section II). The simple Minnaert mechanism IV therefore still remains an adequate explaination for point (i). It is clearly desirable to expand this mechanism, however, to include both the non-hyperbolic dependence of turnover on concentration of cytochrome *c* (ii) and internal events in enzyme turnover. As ably demonstrated by Myers and Palmer [66] proving that a mechanism satisfies both points (i) and (ii) is far from trivial. Equal binding of ferro- and ferricytochrome *c* to the enzyme only ensures first-order cytochrome *c* oxidation kinetics if the Minnaert mechanism is correct (or if a more complex mechanism can be expressed in similar terms). Explanations of the non-hyperbolic dependence of turnover with increasing cytochrome *c* concentrations that rely on negative co-operativity within oxidase dimers appear to be inconsistent with most studies on the oxidase monomer. The question remaining is therefore whether the binding of two cytochrome *c* molecules is required for biphasic Eadie-Hofstee plots to be observed, or whether conformational changes in the oxidase are sufficient. Despite the recent acceptance by several groups of the conformational transition model [40,71,82], I have attempted to show that the original two binding sites model is still a viable alternative. It is difficult to distinguish between these two models merely using kinetic data. Indeed, the current confusion can be illustrated by two recent papers. In one the initial proponent of the controlled binding hypothesis produced a model that required only one cytochrome *c* binding per enzyme [72]; in the other the initial proponent of the single cytochrome *c* binding site model admitted that the only mechanism that currently fit the kinetic data involved binding of two molecules of cytochrome *c* per enzyme [66]. The two competing models both explain the biphasic to monophasic switch at higher ionic strengths, although, interestingly, the conformational transition mechanism predicts the 'low-affinity' reaction is the one observed at higher ionic strengths, whereas the two binding site hypothesis predicts this reaction becomes ineffective.

In the end it may well be that clear biochemical rather than kinetics results will distinguish between the two hypotheses. Thus, the current controversy [71,72] over whether one or two cytochrome *c* molecules are bound to the enzyme during the switch between the high- and low-affinity reactions is clearly in need of resolution. It is also important to remember the data that suggest a catalytic role for the low-affinity site: (i) the reduction of cytochrome *a* (and possibly a_3) in the high-affinity porphyrin cytochrome *c*-cytochrome oxidase complex by cytochrome *c* molecules binding with a low-affinity constant [75] and (ii) the monophasic low-affinity reaction of hexamine ruthenium chloride and yeast cytochrome *c* with PS3 cytochrome *c*-aa_3 [17]. Some re-evaluation of this data is clearly required if the second site is to be treated as regulatory (controlled binding mechanism) or unimportant (conformational transition mechanism). Repeated these measurements whilst monitoring the binding at the low-affinity site with the more sensitive techniques now available [71,72] would be especially helpful.

The pH effects on enzyme turnover are of especial interest given the proton translocation function of the enzyme. Several detailed models have been recently proposed to explain the mechanism of proton translocation [150–155]. It is possible that only when the general mechanism of proton translocation is understood will the steady-state kinetics be amdenable to a full analysis and vice versa. The properties of the complete enzyme cycle may thus be necessary to understand both the steady-state kinetics and proton translocation [154].

The link between proton translocation mechanisms and steady-state kinetics is most clear in studies of enzyme turnover in the presence of a proton motive force, where unfortunately little work has been done as yet. Of course, such a link may be very tenuous if it is discovered that the reactions that control enzyme turnover in the coupled state are not directly related to proton translocation events.

It is also important to reiterate that studies on the isolated oxidase may not be relevant to the enzyme in its physiological state (see section IIIB). For example it is attractive to suppose that the findings by some workers [133,141,143,156] that ΔpH can inhibit oxidase turnover per mV more than $\Delta\Psi$ allows in vivo control of enzyme turnover by both the large $\Delta\Psi$ and the small ΔpH across the inner mitochondrial membrane. If, however, the pH gradient across the inner mitochondrial membrane is much smaller than that seen across the proteoliposomal membrane, the control by ΔpH

seen in proteoliposomes may be of little physiological relevance. Similarly, the attractive proposal that the putative second cytochrome *c* binding site can regulate enzyme turnover in vivo, may not be relevant if the ionic strength of the intracristal space is so high that the second molecule never binds productively. Indeed, the physiological relevance of all the cytochrome *c* oxidation kinetics must be tempered by ignorance of the exact conditions in the intramembrane space. Are ions excluded from this region or is the ionic strength here as high as inside the mitochondria? Is the volume large enough to allow free diffusion or are multi-enzyme complexes de rigueur. Similar questions can be raised about conditions in the periplasmic space [157].

Despite these problems the further study and testing of the kinetics models described here will clearly enrich our knowledge as to the mechanism of this complex multi-subunit, multi-substrate enzyme. Considerable progress has been made in the 15 years since the previous review on cytochrome *c*–cytochrome oxidase interactions [29] and unlike the 1980s [66] it is to be hoped the 1990s will lead to a workable mechanism that accurately depicts the cytochrome oxidase reaction.

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