# Independent Control of Respiration in Cytochrome *c* Oxidase Vesicles by pH and Electrical Gradients†

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ABSTRACT: The effects of altering the pH and electrical components of the membrane potential on the visible spectra and oxygen consumption rates of cytochrome oxidase vesicles were examined during steady-state respiration using cytochrome *c* as the substrate. Heme *a* was found to be 30–55% reduced in the presence of a membrane potential, becoming more reduced when the electrical gradient ($\Delta\psi$) was abolished by valinomycin and more oxidized when the pH gradient ($\Delta$pH) was abolished by nigericin, with little increase (1.2–1.8-fold) in the rates of oxygen consumption in either case. When both gradients were eliminated, heme *a* reduction was close to initial levels, and activity was stimulated up to 8-fold. The magnitude of the changes in heme *a* reduction levels upon elimination of a gradient component was shown to be positively correlated with the magnitude of the respiratory control ratio of the vesicle preparation. Kinetic analysis of the dependence of oxidase activity on cytochrome *c* concentration indicated that changes in the Michaelis constant of the enzyme for its substrate are not a major factor in regulation by either $\Delta$pH or $\Delta\psi$. These results suggest a dual mechanism for respiratory control in cytochrome oxidase vesicles under steady-state conditions, in which the electrical gradient predominantly affects electron transfer from cytochrome *c* to heme *a*, possibly by altering the reduction potential of heme *a*, while the pH gradient affects electron transfer from heme *a* ($Cu_A$) to heme $a_3$ ($Cu_B$), possibly by a conformationally mediated change in the reduction potential of heme $a_3$ or in the kinetics of the electron-transfer process.

Hinkle et al. (1972) demonstrated that cytochrome *c* oxidase (EC 1.9.3.1) incorporated into phospholipid vesicles exhibits respiratory control in a manner apparently similar to that observed in mitochondrial membranes. It can be shown that vesicular cytochrome oxidase generates a transmembrane electrochemical gradient which appears to inhibit turnover rates, resulting in controlled activity. Equilibration of this gradient by addition of ionophores increases electron-transfer activity, giving rise to uncontrolled or "uncoupled" rates. The gradient in right-side-out vesicles, reconstituted such that the enzyme accepts electrons from externally added cytochrome *c*, is positive and acidic on the outside. Various factors have been proposed to be the dominant rate-controlling element of the transmembrane gradient: (1) the lack of substrate protons in the intravesicular space; (2) the electrical component ($\Delta\psi$)[1] of the gradient; or (3) the $H^+$ concentration component ($\Delta$pH) of the proton gradient.

If the concentration of intravesicular protons needed for continued reduction of oxygen to water and for proton translocation were the sole rate-limiting factor (Nicholls, 1982), coupled activity would be a function of the rate at which protons "leak" back into the intravesicular space, and control would depend on the degree of membrane impermeability. However, studies indicate that enhancing the rate of the return of protons to the vesicle interior by adding nigericin does not relieve the control (Moroney et al., 1984; Brunori et al., 1985). In addition, the supply of protons in highly buffered solutions does not appear to limit the reduction of oxygen intermediates in the very high turnover carbonic anhydrase system (Lindskog & Coleman, 1973). This suggests that some aspect of the intrinsic activity of the protein is altered in response to an electrochemical gradient (Brunori et al., 1985). [However, see Konstantinov et al. (1986) for discussion of possible electrogenic control of proton access to the heme $a_3$–$Cu_B$ site.]

Using whole mitochondria, Hinkle and Mitchell (1970) observed that the transfer of electrons from cytochrome *c* to heme *a* was inhibited in the presence of an electrical gradient (positive on the outside) due to an apparent change in the redox potential of heme *a*. In later studies, Brunori et al. (1985) examined the rapid kinetics of cytochrome *c* oxidation by cytochrome oxidase vesicles and found that equilibration of the electrical gradient alone, but not the pH gradient, increased the rate of oxidation of cytochrome *c*. This group concluded that the major regulator of oxidase activity is the electrical component of the gradient and proposed that it functions by mediating a change in protein conformation which alters the kinetics of electron transfer. Similar conclusions have been reached by Azzi and co-workers (Muller et al., 1987).

While the studies discussed above suggest a dominant effect of the electrical gradient in respiratory control, other investigations indicate an important role of the pH gradient in control of respiration in cytochrome oxidase vesicles. Moroney et al. (1984) observed that elimination of the pH gradient decreased the level of steady-state reduction of heme *a*, indicating a release of electron transfer from heme *a* to heme $a_3$. They concluded that pH was controlling this step, in addition to the electrical–potential control of electron transfer between cytochrome *c* and heme *a* demonstrated by Hinkle and Mitchell (1970). Nicholls and co-workers (Shaughnessy & Nicholls, 1985; Nicholls et al., 1988) studied the effects of ionophores on enzyme activity and on the formation of pH

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[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $E_m$, midpoint oxidation–reduction potential; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine; $\Delta$pH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical gradient.

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and electrical gradients in cytochrome oxidase vesicles and concluded that the pH gradient imposed a greater inhibitory effect on steady-state oxidase activity than did the electrical gradient. Control of oxidase activity by pH is also consistent with kinetic studies which show that the maximal velocity of cytochrome oxidase decreases with increasing pH (Wilms et al., 1980; Thornstrom et al., 1984; Gregory & Ferguson-Miller, 1988).

We have investigated changes in the reduction levels of heme *a* and cytochrome *c* in addition to the rates of oxygen consumption and cytochrome *c* oxidation under steady-state conditions following elimination of the pH gradient or the electrical gradient. Our results demonstrate that the predominant control by the pH and electrical gradients is exerted at different steps in the electron-transport sequence of cytochrome oxidase under steady-state conditions.

## Materials and Methods

Most of the studies described in this paper were performed on two types of cytochrome oxidase preparations: a subunit III containing enzyme purified from beef heart according to Suarez et al. (1984) but without the described ethanol wash step and a subunit III depleted form isolated from rat liver according to Thompson and Ferguson-Miller (1983). These preparations typically differ from each other in respiratory control ratios (RCR) following reconstitution: RCR = 5–8 and RCR = 8–15, respectively. Several other preparations were used where indicated in addition to the two already described: (1) a preparation of beef heart oxidase from which subunit III was partially removed (75% depleted) according to the method of Hill and Robinson (1986) (RCR = 9–14); (2) a preparation of rat liver oxidase containing about 30% subunit III, obtained by the procedure of Thompson and Ferguson-Miller (1983) omitting the hydroxylapatite chromatography (RCR = 8–9); and (3) another preparation of beef heart oxidase (subunit III containing) purified from "green fraction" [a product of the fractionation of beef heart mitochondria (Hatefi, 1978), a generous gift from Dr. Y. Hatefi] according to the method of Suarez et al. (1984) starting at the ethanol wash step (RCR = 2–4).

Cytochrome *c* (horse heart, Sigma type VI) was purified as described by Brautigan et al. (1978) followed by gel chromatography of the reduced form on Sephadex G-50-40 in 40 mM KCl/10 mM Hepes–KOH (pH 7.4) to remove polymerized forms and excess dithionite. Sources of other materials are listed in Thompson and Ferguson-Miller (1983).

Cytochrome oxidase was incorporated into asolectin vesicles by using a modification of the cholate dialysis procedure described by Casey et al. (1979). Soybean phospholipids [purified as described by Gregory and Ferguson-Miller (1988)] were sonicated for 1 min/mL in 2% sodium cholate and 75 mM Hepes–KOH, pH 7.4. Enzyme was added at a ratio of 0.1 nmol of $aa_3$/mg of phospholipid, and the mixture was dialyzed 36–42 h against four changes of buffers of constant pH and potassium concentration. The final buffer was 10 mM Hepes–KOH (pH 7.4)/40 mM KCl/38 mM sucrose. The majority of the oxidase molecules (80–90%) were found to be oriented with the cytochrome *c* binding site on the outside of the vesicles, as determined from the fraction that could be reduced by externally added cytochrome *c* and ascorbate.

Enzyme activities and respiratory control ratios were measured polarographically (Thompson & Ferguson-Miller, 1983) or spectrophotometrically in buffer identical with the last dialysis solution. Specific conditions are described in the figure legends. Turnover rates from spectroscopic assays were determined by calculating the initial rate using the integral method described by Yonetani and Ray (1965) and then dividing by the enzyme concentration.

Steady-state kinetic analysis of the dependence of oxidase activity on cytochrome *c* concentration was carried out under the conditions described in the legend to Figure 6. The data were analyzed according to the formula of Sinjorgo et al. (1984), assuming a two-phase mechanism. A computer program was employed that used an iterative procedure to correct each phase for the contribution of the other until the best fit to the data was achieved.

Steady-state reduction spectra of heme *a* and cytochrome *c* were recorded with an Aminco DW2a spectrophotometer using 1-cm path-length cells. Assays were performed in buffer identical with the last dialysis solution to avoid gradients generated by nonenzymatic mechanisms. Cytochrome *c* was added at levels which support sufficient oxidase activity to create inhibitory transmembrane gradients but not so much as to obscure the spectral signal of heme *a* and drive high levels of activity that would too quickly consume available oxygen. Ascorbate and TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) were added to keep the substrate reduced. The ratios of enzyme to substrate used (3–4 mol of cyt *c*/mol of heme $aa_3$) in the measurements of steady-state reduction levels, although not saturating for uncoupled respiration, allowed 3–8-fold increases in activity upon uncoupling. Other conditions are described in the figure legends. Heme *a* was assumed to contribute 100% of the absorbance at 605 nm minus 630 nm, under steady-state conditions where $a_3$ is oxidized (Gibson et al., 1965), and 80% of the absorbance after complete reduction [see Wikstrom et al. (1981)]. The reduction of cytochrome *c* was measured by the absorbance at 550 nm minus 542 nm. Heme $aa_3$ and cytochrome *c* were considered completely reduced 5 min after the cell became anaerobic.

In the steady-state difference spectra (steady state minus oxidized) of the cytochrome oxidase vesicles when coupled or when nigericin was added, red shifts were observed in the 605-nm peak of heme $aa_3$ and the 550-nm peak of cytochrome *c*. In addition, we found anomalous absorption in the 630-nm region which largely disappeared upon addition of valinomycin or upon anaerobiosis. It appears that a small amount of $TMPD^+$ (Wurster's blue, resulting from oxidation of TMPD) electrophoretically migrates to the vesicle interior where it cannot be re-reduced by external ascorbate and thus contributes to the absorbance spectrum (Nicholls et al., 1988). If the absorbance of $TMPD^+$ in the 500–650-nm region is subtracted in proportion to the absorbance observed at 630 nm, the cytochrome oxidase peaks appear in their normal position and reduction levels of heme *a* and cytochrome *c* can be determined.

## Results

In our effort to determine the role of subunit III in the activity of cytochrome oxidase, we have studied the effect of subunit III depletion on the pH dependence of kinetic parameters and on control of activity by transmembrane gradients (Gregory & Ferguson-Miller, 1988). Interpretation of the results of the latter study was complicated by the conflicting results and controversy surrounding the relative contributions of components of the membrane potential to respiratory control in cytochrome oxidase vesicles. The following experiments were designed to clarify the regulatory roles of the electrical and pH components of the potential and thus allow a more meaningful evaluation of the role of subunit III. Our conclusions regarding this particular subunit have been reported (Gregory & Ferguson-Miller, 1988): the same qualitative relationship between the membrane potential and

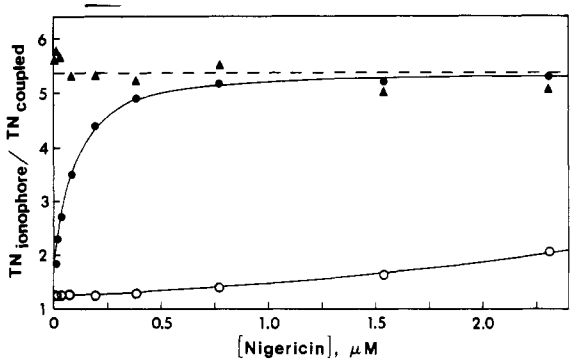


FIGURE 1: Stimulation of enzyme activity in cytochrome oxidase vesicles versus nigericin concentration. Turnover numbers (TN) for reconstituted subunit III containing beef heart oxidase (0.03 nmol of $aa_3$) were calculated from rates of oxygen consumption at 25 °C in 1.75 mL of 40 mM KCl/10 mM Hepes-KOH (pH 7.4)/38 mM sucrose in the presence of 35 μM cytochrome *c*, 5.6 mM ascorbate, and 0.28 mM TMPD. Ionophore-induced stimulation is represented as the ratio of stimulated activity divided by unstimulated activity ($TN_{ionophore}/TN_{coupled}$): with increasing concentrations of nigericin (○); upon addition of valinomycin (1.4 μM; ●), and after the addition of CCCP (6.9 μM; ▲). In these assays, 1 μM nigericin corresponds to 4.4 nmol of nigericin/mg of phospholipid.

oxidase activity was observed with and without subunit III, and any quantitative differences were correlated with the degree of respiratory control exhibited by the vesicle preparation rather than the subunit III content of the enzyme. The studies reported here include results from both III-containing and III-depleted oxidase preparations as indicated, since the different degrees of respiratory control obtained with different preparations provided a useful variable for analyzing the control response.

*Independent Elimination of Δψ and ΔpH.* Valinomycin, a potassium ionophore, has been shown to equilibrate transmembrane electrical gradients with some increase in ΔpH, while nigericin, a proton/potassium exchanger, equilibrates the pH gradient with a concomitant increase in the electrical gradient (Singh & Nicholls, 1985; Nicholls et al., 1988). Thus, independent elimination of the Δψ and ΔpH can be achieved by addition of the appropriate ionophore. However, each of these ion carriers becomes less specific in its behavior at higher concentration (Ferguson et al., 1971; Pressman, 1976). In addition, there are some conflicting reports regarding whether one or the other of these compounds more effectively stimulates oxidase activity when added to proteoliposomes (Brunori et al., 1985; Shaughnessy & Nicholls, 1985), including results suggesting that valinomycin inhibits oxidase activity (Nicholls et al., 1988). Therefore, preliminary to other studies, we determined the minimum concentrations of nigericin and valinomycin needed in combination to obtain maximal enzyme stimulation (uncoupling) for a particular set of conditions. For example, Figure 1 shows the stimulation of activity upon addition of increasing amounts of nigericin in the absence and presence of a fixed amount of valinomycin and compares it to the maximum stimulation achieved by adding CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). When nigericin is added alone at concentrations insufficient to fully release respiratory control even in the presence of valinomycin, activity is increased by 20%, and as concentrations begin to exceed the amount needed to uncouple vesicles when valinomycin is present, activity is stimulated to a greater degree, presumably representing equilibration of both the ΔpH and Δψ by an electrogenic mechanism (Ferguson et al., 1971; Pressman et al., 1976). Nigericin at 0.8 μM (or 3.5 nmol/mg of phospholipid) was used to preferentially equilibrate pH in subsequent experiments using this vesicle preparation. The minimal

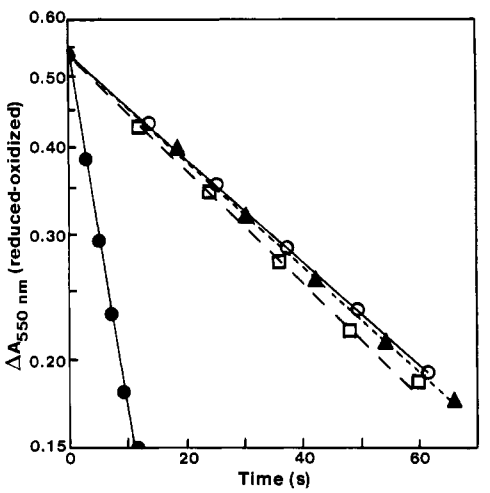


FIGURE 2: Effect of independent elimination of Δψ and ΔpH on oxidation of ferrocytochrome *c* by cytochrome oxidase vesicles: semilogarithmic plots of ferrocytochrome *c* concentration versus time of reaction. The absorbance of 26.9 μM cytochrome *c* (reduced minus oxidized) in 10 mM Hepes-KOH (pH 7.4)/40 mM KCl/38 mM sucrose at room temperature was monitored at 550 nm (path length of 1 cm) after addition of cytochrome oxidase vesicles (16 nM subunit III containing beef heart $aa_3$). Assays contained no ionophores (○), 1 μM valinomycin (i.e., 5 nmol/mg of phospholipid, ▲), 0.2 μM nigericin (i.e., 1 nmol/mg of phospholipid, □), or 1 μM valinomycin and 0.2 μM nigericin (●). An RCR of 6.3 was calculated from this assay.

concentrations of valinomycin required were determined in a similar manner. The optimal ionophore concentrations varied somewhat between vesicle preparations, and while the levels required increased with the total lipid content of the assay (as might be expected for these hydrophobic reagents), this relationship was not linear (lower ionophore to lipid ratios were needed when lipid concentration was high).

Using the appropriate minimal concentrations, we found that the addition of valinomycin alone or nigericin alone stimulated electron-transfer activity in cytochrome oxidase vesicles only slightly (1.2–1.8-fold) and that both were needed to completely release respiratory control, thus allowing 5–17-fold increases in rates of oxygen consumption. We found this to be true whether steady-state activities were measured polarographically as rates of oxygen consumption (with ascorbate and TMPD added as reductants) or spectrophotometrically as rates of ferrocytochrome *c* oxidation (Figure 2). In the latter case, no artificial reducing agents were present that might affect the rate-limiting steps in the steady-state activity.

*Changes in Steady-State Reduction of Heme a and Cytochrome c in Response to Alterations in the Membrane Potential.* To investigate the nature of the regulatory effects of individual membrane potential components on the activity of cytochrome oxidase, we monitored changes in reduction of heme *a* and cytochrome *c* in response to specific elimination of Δψ or ΔpH by ionophores. Previous studies have examined the changes between coupled and uncoupled vesicles (Wrigglesworth & Nicholls, 1978) or the changes following specific equilibration of Δψ or ΔpH but using hexaammineruthenium(II) as an artificial substrate (Moroney et al., 1984). In our studies, low levels of the natural substrate cytochrome *c* were used in conjunction with ascorbate and TMPD.

Starting with coupled vesicles, the transitions in steady-state reduction levels of heme *a* and cytochrome *c* upon equilibration of the electrical gradient (using valinomycin) and the total gradient (using valinomycin and CCCP) are shown in Figure 3A. The spectrum of the completely reduced system (anaerobic) is included for comparison. Heme *a* was found to be 30–55% reduced in the coupled state while cytochrome

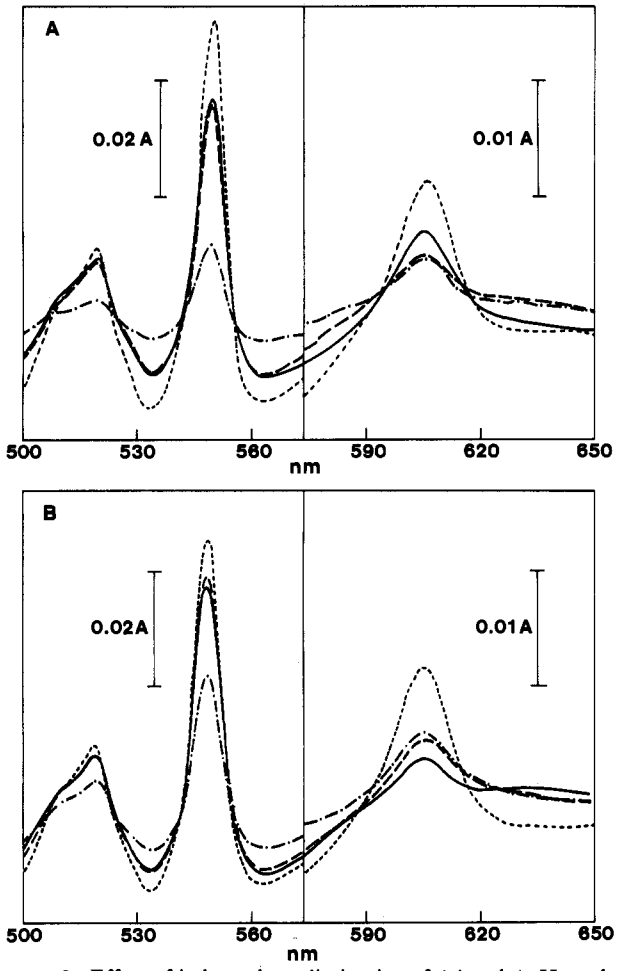


FIGURE 3: Effect of independent elimination of $\Delta\psi$ and $\Delta$pH on the reduction of heme *a* and cytochrome *c*: difference spectra (steady state minus oxidized) of cytochrome oxidase vesicles. Cytochrome oxidase vesicles (III-containing beef heart oxidase) were diluted to approximately 0.6 $\mu$M $aa_3$ in 10 mM Hepes–KOH (pH 7.4)/40 mM KCl/38 mM sucrose, and then cytochrome *c* (2 $\mu$M final) was added. The suspension was maintained at 18 °C during assay. Base-line spectra (oxidized versus oxidized) were subtracted from steady-state spectra. The steady-state spectra of coupled vesicles (– –) were obtained in the presence of 12 mM ascorbate and 0.3 mM TMPD. In (A), the electrical gradient was abolished by the addition of 4 $\mu$M valinomycin (0.7 nmol/mg of phospholipid, —), and in (B), the pH gradient was abolished by the addition of 5.5 $\mu$M nigericin (0.9 nmol/mg of phospholipid, —). The remaining pH gradient in (A) was equilibrated by adding 20 $\mu$M CCCP (–·–), and the remaining electrical gradient in (B) was equilibrated by adding 4 $\mu$M valinomycin (–·–). Spectra were also recorded 5 min after anaerobiosis in each system (– – –).

*c* was 70–90% reduced. After equilibration of $\Delta\psi$, the fraction of total heme *a* reduced increased to 60–80%, while there was very little change (<5%) in the level of reduction of cytochrome *c*. These changes occurred within 20 s of the addition of valinomycin. Only slight increases in enzyme turnover were observed at this stage (1.3–1.7-fold). This result suggests that the electrical gradient exerted some regulation on the transfer of electrons from cytochrome *c* to heme *a* that was released upon addition of valinomycin but that electron flow remained inhibited beyond heme *a*. When CCCP (a protonophore) or nigericin plus valinomycin (data not shown) was present to uncouple the vesicles, both heme *a* and cytochrome *c* became more oxidized, and activity increased 3–8-fold, indicating that elimination of the pH gradient released control on electron transfer within the enzyme.

The alterations in redox levels when the pH gradient is equilibrated first are shown in Figure 3B. When nigericin was

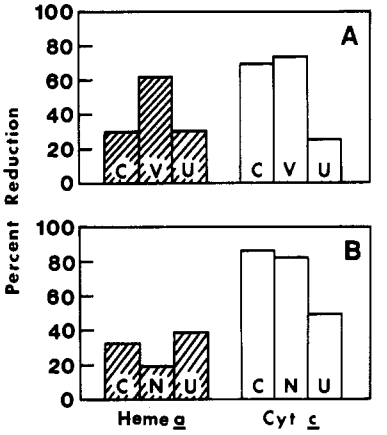


FIGURE 4: Changes in levels of reduction of heme *a* (hatched bars) and cytochrome *c* (open bars) upon independent equilibration of $\Delta\psi$ and $\Delta$pH. (A) Equilibration of the electrical potential followed by the pH gradient (as in Figure 3A). (B) Equilibration of the pH gradient followed by the electrical gradient (as in Figure 3B). Percent reduction of cytochrome *c* and heme *a* in cytochrome oxidase vesicles is shown for (C) the coupled state, (V) after elimination of only the $\Delta\psi$ by addition of valinomycin, (N) after elimination of only the $\Delta$pH of the addition of nigericin, and (U) after the vesicles were uncoupled by equilibration of both components of the proton electrochemical gradient. Levels of reduction were calculated as percent of the totally reduced system upon anaerobiosis, as described under Materials and Methods. The levels of reduction and the magnitudes of the changes agree within 10% upon replication for a given vesicle preparation, but the sizes of the changes observed in different vesicle preparations are dependent on the RCR (See Figure 5).

added to coupled vesicles to eliminate the $H^+$ concentration gradient, heme *a* became more oxidized while the reduction level of cytochrome *c* was unchanged. Oxygen consumption increased only slightly. This suggests that removal of the pH gradient releases electron transfer between heme *a* and $a_3$ but that the electrical gradient is still inhibiting the input of electrons from cytochrome *c*. The levels of heme *a* reduction increased when valinomycin was added to these pH-equilibrated vesicles and cytochrome *c* became more oxidized. This was accompanied by full stimulation of activity.

Figure 4 is a summary of the changes in levels of reduction of heme *a* and cytochrome *c* in the same vesicle preparation with sequential equilibration of gradient components. The independent elimination of $\Delta\psi$ or $\Delta$pH causes significant changes in the reduction level of heme *a*, but they are of opposite direction apparently due to the differential release of control of electron flow to or away from this redox center (i.e., from cytochrome *c* to heme *a* and from heme *a* to heme $a_3$, respectively). Reduction of cytochrome *c*, however, is largely unaffected by the elimination of only one gradient component, since enzyme turnover increases very little. The level of reduction of heme *a* in the uncoupled state returns to a level close to, but usually more reduced than, that of the coupled state, while cytochrome *c* is significantly more oxidized in the uncoupled state, reflecting full stimulation of activity under these substrate-limited conditions. This is compatible with the results of Wrigglesworth and Nicholls (1978), who observed a slight increase in reduction of heme *a* along with a substantial oxidation of cytochrome *c* when the steady-state spectra of controlled and uncontrolled vesicles were compared.

The respiratory control ratio (RCR) is used as an indicator of the responsiveness of cytochrome oxidase in vesicles to a membrane potential. It presumably reflects the degree of correct insertion and the level of the membrane potential developed in steady state. We tested various types of enzyme preparations exhibiting differing respiratory control ratios for responses to alterations in the membrane potential. Figure

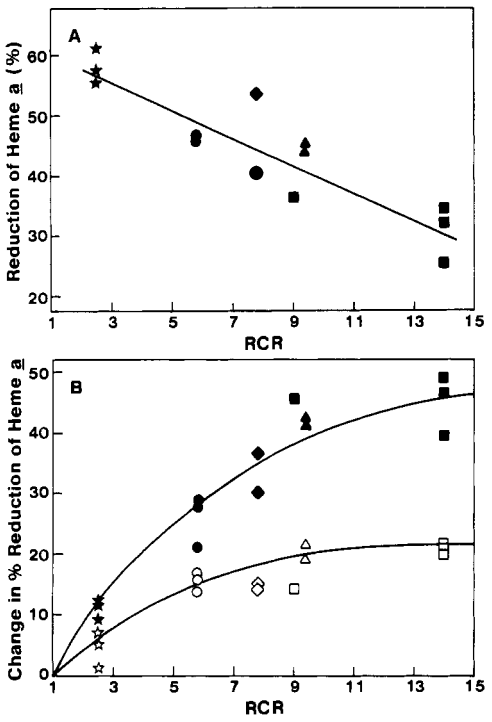


FIGURE 5: (A) Relationship between respiratory control ratios (RCR) and the percent of heme *a* reduced in the coupled state (no ionophores). (B) Relationship between respiratory control ratios (RCR) and the magnitude of the changes in percent reduction of heme *a* upon addition of ionophores. In (B), the solid symbols represent the increase in percent reduction upon elimination of the electrical potential by adding valinomycin to coupled vesicles, and the open symbols represent the *decrease* in percent reduction (plotted as a positive value) when electrically equilibrated vesicles become completely uncoupled by the addition of CCCP. The reduction of heme *a* was monitored at 605 minus 630 nm under the conditions described in the legend of Figure 3. A range of RCR values was achieved by reconstituting enzyme preparations that typically vary in this characteristic: subunit III containing beef heart oxidase (●, ○); rat liver oxidase, 95–98% depleted of subunit III (■, □); beef heart oxidase, 75% depleted of subunit III (▲, △); rat liver oxidase, 70% depleted of subunit III (♦, ◇); and subunit III containing beef heart oxidase prepared from "green fraction" by a method involving an ethanol wash (★, ☆). See Materials and Methods for a more detailed description of oxidase preparations and for the method of RCR determination.

5A shows the correlation between RCR and the percent of heme *a* reduced in the coupled state. The high levels of reduction with lower respiratory control may reflect the partial release of (or inability to maintain) the electrical gradient. Upon elimination of gradient components, we found that all vesicle preparations showed the same qualitative changes in heme *a* reduction (an increase upon addition of valinomycin followed by a decrease upon addition of CCCP) and the magnitude of the change was dependent on the magnitude of the respiratory control ratio (Figure 5B), indicating the relationship between these redox changes and the control of activity.

*Effect of Δψ and ΔpH on Steady-State Kinetics of Cytochrome Oxidase.* To determine whether the inhibition of oxidase activity by either of the membrane potential components could be accounted for by a change in affinity for substrate, we studied the separate and combined effects of nigericin and valinomycin additions on rates of oxygen consumption as a function of cytochrome *c* concentration.

Analysis of the data by Eadie–Hofstee plots reveals that the kinetics of the vesicular enzyme with cytochrome *c* are biphasic in coupled, partially equilibrated, and uncoupled states. Figure 6 shows the data for subunit III containing beef heart oxidase vesicles. Assuming that the kinetic behavior is the result of

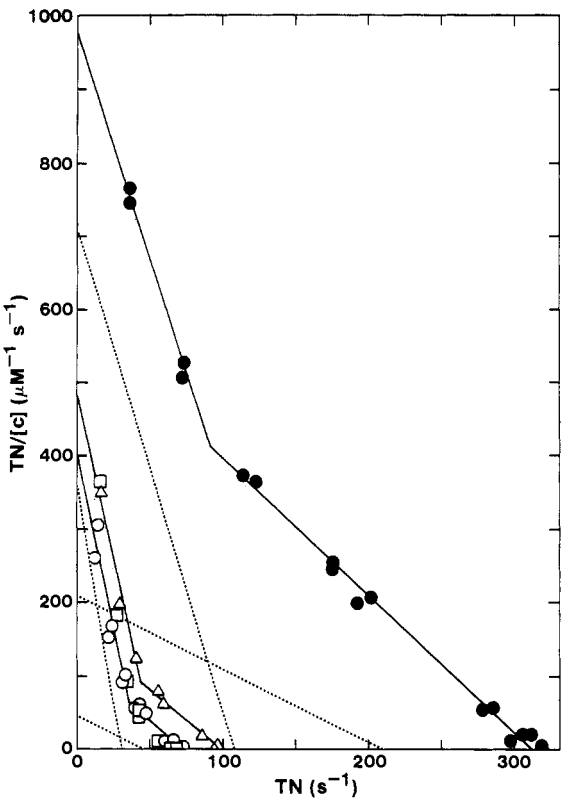


FIGURE 6: Eadie–Hofstee plots of activity of reconstituted cytochrome oxidase (subunit III containing enzyme from beef heart) under coupled and uncoupled conditions. Coupled activity (○) in absence of ionophores; valinomycin-stimulated activity (□); nigericin-stimulated activity (△); uncoupled activity (●) with nigericin plus valinomycin. Conditions as described in the legend to Figure 1 with 0.04 nmol of $aa_3$, 1.1 μM valinomycin (4.8 nmol/mg of phospholipid), and 0.8 μM nigericin (3.5 nmol/mg of phospholipid). The range of cytochrome *c* concentrations was from 0.1 to 33 μM. The dotted lines indicate the two components of the coupled and uncoupled kinetic curves which were derived by computer analysis as described under Materials and Methods.

at least two separate interactions of cytochrome *c* with the oxidase, it can be analyzed in a manner described under Materials and Methods to yield kinetic constants for the two phases (for coupled, $TN_{max1} = 27\ s^{-1}$, $K_{m1} = 7 \times 10^{-8}$ M, $TN_{max2} = 44\ s^{-1}$, and $K_{m2} = 1 \times 10^{-6}$ M; for uncoupled, $TN_{max1} = 110\ s^{-1}$, $K_{m1} = 15 \times 10^{-8}$ M, $TN_{max2} = 210\ s^{-1}$, and $K_{m2} = 1 \times 10^{-6}$ M). Although these values give a good fit to the kinetic curves, they must be considered "apparent" constants because of the uncertainty concerning the number and nature of the interactions of cytochrome *c* with cytochrome oxidase (Ferguson-Miller et al., 1976; Errede & Kamen, 1978; Antalis & Palmer, 1982; Speck et al., 1984; Sinjorgo et al., 1984). Nevertheless, a strong correlation between the apparent $K_m$ values and the binding constants for cytochrome *c* has been shown (Ferguson-Miller et al., 1976, 1979). In view of this, comparison of the $K_m$ values for the coupled and uncoupled states may give some information about the effect of the membrane potential on the binding affinity of cytochrome *c*. In fact, the apparent $K_m$ values for the low-affinity phase were relatively unaffected by the partial or complete removal of the transmembrane gradient, while those for the high-affinity reaction were somewhat lower (approximately 2-fold) for the coupled compared to the uncoupled state. While these changes in $K_m$ values cannot be interpreted unequivocally, the results suggest that, if anything, there is an increase rather than a decrease in affinity for cytochrome *c* in the presence of a membrane potential, which would not be expected to contribute to control of electron transfer from cytochrome *c* to heme *a*.

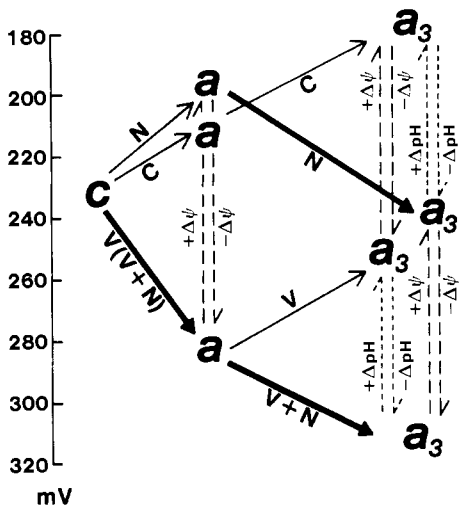


FIGURE 7: Hypothetical scheme for the dual control of respiration in cytochrome oxidase vesicles by the electrochemical gradient. See Discussion for detailed description. Redox centers [cytochrome *c* (*c*); heme *a* (*a*); and heme $a_3$ ($a_3$)] are arranged according to midpoint potentials ($E_m$, scale at left) in the presence and absence of ΔpH and Δψ. Controlled electron-transfer steps (light arrows); uncontrolled electron-transfer steps (bold arrows); changes in transmembrane gradients (dashed arrows). Path of electron transport: in controlled conditions (C); in presence of nigericin (N); and in the presence of valinomycin (V).

## DISCUSSION

The lack of any significant stimulation in oxygen consumption rates upon addition of nigericin to cytochrome oxidase vesicles demonstrates that (1) enzyme activity in coupled vesicles is not simply limited by a lack of substrate protons in the vesicle interior bulk phase and (2) elimination of the pH gradient alone cannot relieve the inhibitory effect on the enzyme. Similarly, release of the electrical gradient by valinomycin alone has little power to increase enzyme turnover rates. When these two ionophores are used in combination, however, the enzyme becomes fully stimulated. These results show that each of the two gradients has the ability to regulate activity. In addition, kinetic analysis of activities in the absence and presence of ionophores indicates that control is not mediated by decreasing the affinity of the enzyme for substrate. Our studies of ionophore-induced alterations in steady-state heme *a* reduction suggest that the electrical gradient predominantly regulates electron transfer from cytochrome *c* to heme *a* while the pH gradient controls the heme *a* to $a_3$ transfer. Our conclusions are consistent with those discussed by Papa (1988).

Figure 7 depicts a model that attempts to explain our observations in terms of a thermodynamic mechanism of regulation in which the controlling forces are represented entirely in terms of changes in reduction potentials of the heme groups. In reality, the control may reflect one of several response mechanisms: (1) a real change in the redox potential resulting from some change in protein structure in the immediate environment of the heme; (2) an apparent change in the redox potential resulting from an altered contribution of the membrane potential to the driving force perceived by the electron; or (3) a real change in the kinetics of electron transfer due to an altered pathway (length, chemical nature) through which the electron must travel. Both (1) and (3) would require some conformational change in the protein, while (2) would simply reflect the degree of electrogenicity of the electron-transfer step.

Since there is considerable variation in the redox potential values reported in the literature, we have chosen to represent the redox potentials of the heme centers with values from references that used conditions most relevant to the reconstituted system at steady state. Our intent is to emphasize relative changes that may correspond to regulatory responses rather than specific values.

Considering first the effects of Δψ, the reduction potential of cytochrome *c* is apparently insensitive to the electrochemical gradient (Wikstrom et al., 1976) but does shift from 280 to 230 mV upon binding (Dutton et al., 1970). Evidence for electrical gradient dependent shifts in the reduction potential of heme *a* was provided by Hinkle and Mitchell (1970) in studies using whole mitochondria. They concluded that the midpoint potential of heme *a* was modified by the electrical gradient in a continuous manner according to the equation:

$$E_m'' = E_m' - 0.5\Delta\psi \quad (1)$$

where a positive Δψ reflects an electric potential that is positive on the outside of the membrane and $E_m'$ and $E_m''$ are the midpoint potentials at electrical gradients of zero and some finite value, respectively. Thus, if the redox potential of heme *a* in the uncontrolled state is 285 mV [as calculated by Wikstrom et al. (1976) for uncoupled mitochondria in aerobic steady state] and the Δψ across the membrane in oxidase vesicles is approximately 150 mV in the coupled state and 175 mV where nigericin is present [as reported by Singh and Nicholls (1985)], the $E_m''$ values would be shifted to about 210 and 198 mV, respectively. This $E_m$ value for heme *a* in the coupled state corresponds well to the 212-mV potential obtained from mitochondria in state 4 respiration (Wikstrom et al., 1976). The reduction potential of $Cu_A$ has been shown to be about 20 mV more positive than that of cytochrome *c* and to be insensitive to the presence of a membrane potential (Rich et al., 1988). Although there is some question whether cytochrome *c* transfers electrons initially to $Cu_A$ (Rich et al., 1988) or heme *a* [see Wikstrom et al. (1981)], our model can accommodate either sequence. (Hence, $Cu_A$ is not depicted in this model.) The redox potentials shown for heme $a_3$ in the coupled and electrically equilibrated states are represented as somewhat less positive than for heme *a* (Dutton & Wilson, 1974; Wikstrom et al., 1976), although more positive values for heme $a_3$ have been reported for other conditions [e.g., see Wilson and Dutton (1970), Dutton et al. (1970), and Wilson et al. (1972)]. A shift in $E_m$ similar to that proposed for heme *a* is suggested for heme $a_3$ upon equilibration of the electrical potential; however, the relatively slight increase in oxidase activity observed upon addition of valinomycin may indicate a smaller alteration in this center. Our observation that the steady-state level of reduction of heme *a* is higher in the absence of an electrical gradient is consistent with an enhancement of the cytochrome *c* to heme *a* electron transfer resulting from a change in reduction potential. The electrical gradient dependence of the redox potential of heme *a* has been demonstrated in mitochondria (Hinkle & Mitchell, 1970; Rich et al., 1988), and our observations on oxidase vesicles provide the additional evidence that the redox state of heme *a* is correlated with the degree of respiratory control.

Another mechanism of Δψ control has been proposed by Wikstrom (1989), involving anticooperative interaction of heme *a* and $Cu_B$ and stabilization by the electrical gradient of a hypothetical dipole created by reduction of $Cu_B$. This model would also predict an increase in the redox potential of heme *a* upon removal of the electrical gradient. Although there has been recent speculation about a role of $Cu_B$ in regulation of oxidase activity (Wikstrom, 1989; Nicholls, 1989), direct evidence regarding its redox state and kinetics of reduction has been difficult to obtain.

A different mechanism of regulation by the electrical gradient has been suggested (Konstantinov et al., 1986) whereby the presence of Δψ impedes access of protons to heme *a* and/or heme $a_3$. If protons are required for heme *a* reduction, removal of Δψ would increase its apparent redox potential.

Considering the effects of ΔpH, the control of electron transfer from heme *a* to heme $a_3$ is also illustrated as a shift in reduction potential, but specifically at heme $a_3$. The pH dependence of the midpoint potential of this center has been documented (Wilson et al., 1972; Blair et al., 1986). However, it is likely that the pH gradient exerts an additional kinetic control of this step by maintaining an alkaline intravesicular pH (Thornstrom et al., 1984; Malmstrom & Andreasson, 1985; Gregory & Ferguson-Miller, 1988). Thus, elimination of the pH gradient by nigericin is represented as causing a slight decrease in the reduction potential of heme *a* due to an increase in Δψ and release of the regulation on transfer from heme *a* to heme $a_3$. The oxidation of heme *a* reflects the increase in transfer from *a* to $a_3$, while the minimal increase in activity reflects the continued block in electron transfer from cytochrome *c* to heme *a*.

The final state represented in the model is that where the addition of valinomycin plus nigericin has removed all regulatory barriers, thermodynamic and/or kinetic.

In studies of changes in oxygen consumption by sonicated proteoliposomes, Nicholls and co-workers (Shaughnessy & Nicholls, 1985; Nicholls et al., 1988) observed that nigericin released respiratory control more effectively than valinomycin. Indeed, these authors found that valinomycin caused an increase in ΔpH and a decrease in enzyme activity and concluded that the ΔpH was the dominant regulatory component in their system. In contrast, we find a small increase in activity with valinomycin addition and an immediate large increase in steady-state reduction of heme *a*. This combination of effects would argue against an increase in ΔpH being the predominant cause of the valinomycin-induced changes that we observe.

Moroney et al. (1984) studied the effects of ionophores on the reduction of heme *a*, in addition to the stimulation of activity of reconstituted oxidase, but in a system substituting hexaammineruthenium for cytochrome *c*. The levels of heme *a* reduction they observed in the coupled state were significantly higher than those we observed (80% versus 35–55%), and although they also found that valinomycin did not stimulate activity, they found that the levels of cytochrome *a* reduction were insensitive to elimination of Δψ by this ionophore. The lack of response to alteration in Δψ in this system can be explained by the low redox potential of hexaammineruthenium (78 mV; Meyer & Taube, 1968) which allows this artificial substrate to drive the reduction of heme *a* in the presence or absence of an electrical gradient and thus bypass the control by the electrical gradient.

In agreement with our findings, Moroney and co-workers observed the oxidation of heme *a* upon elimination of ΔpH. However, their results show that nigericin caused a 3-fold increase in activity when used at a level causing only a 30% increase in our vesicles. Again, due to the low potential of the hexaammineruthenium, this can be predicted by our model since the remaining Δψ in their system would not inhibit the donation of electrons to heme *a* as it would if cytochrome *c* were the donor. Thus, both sets of data demonstrate the regulation of electron transfer from heme *a* to $a_3$ by ΔpH (or the intravesicular pH).

By studying the differential effects of ionophores on the rapid kinetics of cytochrome *c* oxidation by reconstituted cytochrome oxidase, Brunori et al. (1985) found that elimination of the pH gradient by nigericin failed to release oxidase activity, while equilibration of the electrical gradient by valinomycin alone resulted in nearly complete stimulation. They concluded that the electrical component of the gradient constituted the major control on activity in cytochrome oxidase vesicles. However, our results (Figures 2 and 6) show no significant release of activity by valinomycin alone, whether assayed as oxygen consumption rates or by spectral measurement of ferrocytochrome *c* oxidation rates. A possible explanation for this discrepancy would be the lack of maintenance of a pH gradient in the vesicles used for the experiments reported by Brunori and co-workers, due to dialysis or assay conditions, or the nature of the lipid vesicles.

We conclude that the vesicle membrane is not simply a barrier that limits the reentry of substrate protons (for the reduction of oxygen) but a capacitor that sustains transmembrane pH and electrical gradients which directly and differentially affect specific steps in the electron-transfer process of cytochrome oxidase. The reconstituted enzyme provides a valuable system to investigate the structural requirements and regulation of the electron and proton transport mechanisms of cytochrome oxidase and has allowed us to rule out the involvement of subunit III in control of activity by transmembrane gradients (Gregory & Ferguson-Miller, 1988).

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## References

Antalis, T. M., & Palmer, G. (1982) *J. Biol. Chem. 257*, 6194–6206.

Blair, D. F., Ellis, W. R., Jr., Wang, H., Gray, H. B., & Chan, S. I. (1986) *J. Biol. Chem. 261*, 11524–11537.

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978) *Methods Enzymol. 53*, 128–164.

Brunori, M., Sarti, P., Colosimo, A., Antonini, G., Malatesta, F., Jones, M. G., & Wilson, M. T. (1985) *EMBO J. 4*, 2365–2368.

Casey, R. P., Chappell, J. B., & Azzi, A. (1979) *Biochem. J. 182*, 149–156.

Dutton, P. L., & Wilson, D. F. (1974) *Biochim. Biophys. Acta 346*, 165–212.

Dutton, P. L., Wilson, D. F., & Lee, C. P. (1970) *Biochemistry 9*, 5077–5082.

Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.

Ferguson, S. M. F., Estrada-O., S., & Lardy, H. A. (1971) *J. Biol. Chem. 246*, 5645–5652.

Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.

Ferguson-Miller, S., Weiss, H., Speck, S. H., Brautigan, D. L., Osheroff, N., & Margoliash, E. (1979) in *Cytochrome Oxidase* (King, T. E., et al., Eds.) pp 281–292, Elsevier/North-Holland Biomedical Press, Amsterdam.

Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 888–894.

Gregory, L. C., & Ferguson-Miller, S. (1988) *Biochemistry 27*, 6307–6314.

Hatefi, Y. (1978) *Methods Enzymol. 53*, 3-4.
Hill, B. C., & Robinson, N. C. (1986) *J. Biol. Chem. 261*, 15356-15359.
Hinkle, P., & Mitchell, P. (1970) *J. Bioenerg. 1*, 45-60.
Hinkle, P. C., Kim, J. J., & Racker, E. (1972) *J. Biol. Chem. 247*, 1338-1339.
Konstantinov, A., Vygodina, T., & Andreev, I. M. (1986) *FEBS Lett. 202*, 229-234.
Lindskog, S., & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2505-2508.
Malmstrom, B. G., & Andreasson, L. (1985) *J. Inorg. Biochem. 23*, 233-242.
Meyer, T. J., & Taube, H. (1968) *Inorg. Chem. 7*, 2369-2379.
Moroney, P. M., Scholes, T. A., & Hinkle, P. C. (1984) *Biochemistry 23*, 4991-4997.
Muller, M., Labonia, N., Schlapfer, B., & Azzi, A. (1987) in *Cytochrome Systems: Molecular Biology and Bioenergetics* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 239-246, Plenum Press, New York.
Nicholls, D. G. (1982) *Bioenergetics: An Introduction to the Chemiosmotic Theory*, Academic Press, New York.
Nicholls, P. (1989) *Ann. N.Y. Acad. Sci.* (in press).
Nicholls, P., Cooper, C. E., & Kjarsgaard, J. (1988) in *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C. H., Tedeschi, H., Diwan, J. J., & Salerno, J. C., Eds.) pp 311-321, Plenum Press, New York.
Papa, S. (1988) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) pp 707-730, Alan R. Liss, Inc., New York.
Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501-530.
Rich, P. R., West, I. C., & Mitchell, P. (1988) *FEBS Lett. 233*, 25-30.
Shaughnessy, S., & Nicholls, P. (1985) *Biochem. Biophys. Res. Commun. 128*, 1025-1030.
Singh, A. P., & Nicholls, P. (1985) *J. Biochem. Biophys. Methods 11*, 95-108.
Sinjorgo, K. M. C., Meijling, J. H., & Muijsers, A. O. (1984) *Biochim. Biophys. Acta 767*, 48-56.
Speck, S. H., Dye, D., & Margoliash, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 347-351.
Suarez, M. D., Revzin, A., Narlock, R., Kempner, E. S., Thompson, D. A., & Ferguson-Miller, S. (1984) *J. Biol. Chem. 259*, 13791-13799.
Thompson, D. A., & Ferguson-Miller, S. (1983) *Biochemistry 22*, 3178-3187.
Thornstrom, P., Soussi, B., Arvidsson, L., & Malmstrom, B. G. (1984) *Chem. Scr. 24*, 230-235.
Wikstrom, M. (1989) *Ann. N.Y. Acad. Sci.* (in press).
Wikstrom, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, London and New York.
Wikstrom, M. K. F., Harmon, H. J., Ingledew, W. J., & Chance, B. (1976) *FEBS Lett. 65*, 259-277.
Wilms, J., VanRijn, J. L. M. L., & Van Gelder, B. F. (1980) *Biochim. Biophys. Acta 593*, 17-23.
Wilson, D. F., & Dutton, P. L. (1970) *Arch. Biochem. Biophys. 136*, 583-584.
Wilson, D. F., Lindsay, J. G., & Brocklehurst, E. S. (1972) *Biochim. Biophys. Acta 256*, 277-286.
Wrigglesworth, J. M., & Nicholls, P. (1978) *FEBS Lett. 91*, 190-193.
Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 3392-3398.

# Tubulin Dimer Dissociation and Proteolytic Accessibility

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ABSTRACT: The $\alpha$ and $\beta$ subunits of the tubulin dimer each possess a distal C-terminal subtilisin cleavage site which, when cleaved, releases an acidic, small peptide. In addition, each possesses an internal site, cleaved by trypsin in $\alpha$ and chymotrypsin in $\beta$, which connects the amino and carboxyl structural domains. A model of the dimer is presented which suggests that the $\beta$ C-terminal subtilisin site may be more accessible in the monomer than in the dimer. Kinetics of cleavage at this site on the dimer yield straight-line plots of log (undigested fraction) versus time, from which pseudo-first-order rate constants are obtained. Temperature effects on the rate constant are due to changes in the activity of subtilisin, not to temperature-induced unfolding around this site. The rate constant is proportional to the subtilisin/tubulin ratio, whether this is varied by changing the concentration of subtilisin or of tubulin. However, if the rate constant increases due to decreasing tubulin concentration, the extrapolated zero time intercept decreases. The decrease in zero time intercept is interpreted as being due to the appearance of a rapidly digested fraction upon dilution of tubulin. The increase observed in this fast fraction with dilution of tubulin is fully reversible upon reconcentration. It is suggested that this fast fraction represents monomeric $\beta$-tubulin and the concentration dependence of this fast fraction indicates a dissociation constant of about $1.5 \times 10^{-7}$ M.

Tubulin is a heterodimer composed of two quite similar but nonidentical subunits, $\alpha$- and $\beta$-tubulin. These are each composed of an amino-terminal domain and a carboxy-terminal domain whose extreme end is highly charged. The linker region connecting the two domains of $\alpha$-tubulin is split by trypsin (after Arg-339) and that connecting the two domains of $\beta$-tubulin by chymotrypsin (after Tyr-281) (Kirchner & Mandelkow, 1985; Sackett & Wolff, 1986). These cleavage sites are not accessible in microtubules or zinc sheet polymers. This, combined with the geometry of the polymers, indicates that these sites are both contained within the long axis of the polymer protofilament and thus likely exposed on opposite sides