BIOCHEMICAL JOURNAL LETTERS

On the location of the electrogenic step(s) during the process of dioxygen reduction catalysed by cytochrome *c* oxidase

In a recent article (O'Shea *et al.*, 1984), we demonstrated that there is an exponential relationship between the rate of net proton flux to an applied voltage across the membranes of both phospholipid vesicles and mitochondria. Thus, once a critical voltage was attained a large increase of conductance occurred. We suggested that in many ways this phenomenon was reminiscent of the behaviour of Zener diodes. The 'Zener' or 'avalanche' region of the membrane may be considered, therefore, to represent the position on the voltage axis where the conductance rapidly changes. The location of this conductance-switching appeared to depend upon such parameters as the experimental conditions and the type of lipid used. For example, our phospholipid vesicle preparations exhibited the switch around 150 mV, whereas the position of the mitochondrial Zener region was around 160–180 mV.

In an attempt to demonstrate one manifestation of such a conductance-switching, we initiated single turnovers of a suspension of cytochrome *c* oxidase vesicles in the absence and presence of a charge-compensation system (see Fig. 5 of O'Shea *et al.*, 1984).

In the absence of any charge compensation system the addition of ferrocytochrome *c* to the vesicles caused a rapid alkalinization. Our explanation of this phenomenon was based on the electrogenic nature of the cytochrome *c* oxidase reaction such that the short-circuit region (Zener) of the current-voltage relationship was entered during the single turnover event. In support of this we were able to calculate that, if there were no initial charge-compensation effects, then a single turnover of each active enzyme generates a membrane potential of around 300 mV. This places the membrane beyond the Zener region and thus a larger conductance to protons resulted. Such an effect is not observed in bacteria or mitochondria due to the relatively much larger total electrical capacitance of these particles.

Unfortunately, these latter experiments may possibly cause some misunderstanding and we

would like to take this opportunity to clarify our interpretation. The confusion could arise from classical explanations of respiratory control. One would expect that, before the membrane potential could reach the Zener region, further electron transport through the enzyme would have been limited. However from our observations (Fig. 5 of O'Shea *et al.*, 1984) described above this does not appear to occur, i.e. the enzyme apparently turns over as if it were uncoupled. We should however qualify this statement by adding that respiratory control does not occur 'during the first turnover (or part of a turnover) of the enzyme ensemble'.

We should now consider, however, the events which lead up to the condition of controlled respiration. After the first turnover of a population of cytochrome oxidase vesicles in the absence of a charge-compensation system, the induced membrane potential will not have dissipated; thus the next turnover event will have to 'push' against the thermodynamic load of the already existing membrane potential. Eventually after these first oscillatory steps the system will attain the quasi-equilibrium controlled state known as 'static head'. This latter condition will be a thermodynamic/kinetic compromise between the magnitude of the steady-state membrane potential which is developed, the rate of dissipation of this potential and the rate of oxidation of ferrocytochrome *c*. Our measurements (Fig. 5 of O'Shea *et al.*, 1984) therefore are not in conflict with the classical explanations of respiratory control, they are merely observations of a state which preludes it.

Although cytochrome *c* oxidase catalyses the four-electron reduction of molecular oxygen to water, which was the criterion for a single turnover discussed above, the enzyme also catalyses the oxidation of individual molecules of ferrocytochrome *c*, and four such oxidation turnovers are required for each molecule of oxygen reduced to water. Thus, applying the same assumptions as before (O'Shea *et al.*, 1984), it is possible to deduce the magnitude of the membrane potential as a consequence of various numbers of turnovers of cytochrome *c* oxidase vesicles (Table 1).

In view of these calculations, respiratory control should be exerted after the first couple of ferrocytochrome *c* oxidation events (0.25–0.5 turnovers) rather than after integral numbers of turnovers of the enzyme. One would expect that even after the

Table 1. *Magnitude of the membrane potential as a consequence of various numbers of turnovers of cytochrome c oxidase vesicles*

Number of turnovers	Membrane potential developed (V)
0.1	0.030
0.25	0.076
0.5	0.152
0.75	0.228
1.0	0.304
2.0	0.608
5.0	1.525
10.0	3.050

oxidation of only one molecule of ferrocycytochrome *c* the membrane potential (approx. 75 mV) would begin to inhibit the oxidation of the next molecule. Furthermore, as this potential does not cause a significant change of the membrane permeability (O'Shea *et al.*, 1984) it would be expected to have a longer lifetime relative to the larger potentials. A similar argument may be applied if two oxidation events were to occur.

The question to which we should address ourselves, therefore, is why do we not see respiratory control after a half/quarter of a turnover of the enzyme, which would manifest itself as a relatively slow rate of alkalization upon addition of ferrocycytochrome *c* in the absence of a charge-compensation system? One possible solution to this enigma would be that the enzyme is not electrogenic for the first half of its catalytic process. Or, in other words, the first two electrons into the enzyme do not cross the membrane but remain outside the vesicle, i.e. there is no charge separation across the membrane. Presumably the electron(s) would be located in the low-potential haem/copper redox cluster, for there is much evidence as to the extramitochondrial/vesicular location of this metal cluster (Wikström *et al.*, 1981). These authors also suggest that this metal cluster is associated with the proton-pumping (electrogenic) function of the enzyme. This suggestion is not at variance with our proposal or observation as, although this centre may indeed abstract a proton from the intra-compartmental phase, the electron is also in very close proximity and, therefore, no charge separation occurs across the membrane. With the subsequent oxidation of the low-potential metal cluster by the high-potential haem/copper centre, however, the proton may somehow (see Wikström *et al.*, 1981) be left facing the extracompartamental phase. Thus, in the presence of a charge-compensation system, this proton may move into the bulk phase and consequently be observed.

To complicate matters, however, we have also observed that the phenomenon of a rapid alkalization upon the addition of ferrocycytochrome *c* in the absence of any charge-compensation system does not always occur in the same manner. It was found that the experimental conditions had a marked effect upon the rate of the alkalization. This may be ascribed to an effect on the current-voltage curve (O'Shea *et al.*, 1984), or an alternative explanation (for which we have some evidence) could be that the enzyme itself is affected by these conditions. Clearly more experimentation is required.

A final point of more general interest relates to earlier studies with cytochrome oxidase vesicles. The extravesicular acidification (as a result of the turnover of the vesicles) decayed to electrochemical equilibrium more than one order of magnitude more slowly than an equivalent pH-jump made with an addition of acid or base (see Casey & Azzi, 1983). More recently (Casey *et al.*, 1984; Thelen *et al.*, 1984) we have attributed such a phenomenon to a suboptimal charge-compensation system present in these earlier experiments. It was shown that in such experiments there exists a small but influential membrane potential which would increase proportionally as more turnovers were initiated. At some point, therefore, respiratory control would become significant and thus the alkalization decay kinetics would be difficult to interpret. It is for this reason that the H^+/e^- stoichiometry apparently decreased when higher numbers of turnovers were initiated (Casey *et al.*, 1979). Thus, great care must always be exercised to quench membrane potentials when making such studies as, depending upon the circumstances, the presence of a membrane potential may either increase or decrease the observed rate of relaxation of 'translocated' protons and thus confuse the investigator.

Paul S. O'SHEA and Angelo AZZI
*Medizinisch-Chemisches Institut, Universität Bern,
Bern, Switzerland*

(Received 6 June 1984)

- Casey, R. P. & Azzi, A. (1983) *FEBS Lett.* **154**, 237–241
Casey, R. P., Chappell, J. B. & Azzi, A. (1979) *Biochem. J.* **182**, 149–156
Casey, R. P., O'Shea, P. S., Chappell, J. B. & Azzi, A. (1984) *Biochim. Biophys. Acta* **765**, 30–37
O'Shea, P. S., Petrone, G., Casey, R. P. & Azzi, A. (1984) *Biochem. J.* **219**, 719–726
Thelen, M., O'Shea, P. S., Petrone, G. & Azzi, A. (1984) *J. Biol. Chem.*, in the press
Wikström, M., Krab, K. & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, London