Use of Oxonol V as a Probe of Membrane Potential in Proteoliposomes Containing Cytochrome Oxidase in the Submitochondrial Orientation†

Chris E. Cooper,‡ Doug Bruce, and Peter Nicholls*

Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada L2S 3A1

Received September 14, 1989; Revised Manuscript Received December 18, 1989

ABSTRACT: Absorbance changes in the anionic dye bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol (oxonol V) can be used to monitor the membrane potential of liposomes and cytochrome *c* containing cytochrome oxidase proteoliposomes (*c*-loaded COV). Diffusion potentials (positive inside the vesicles) cause an increase in the dye extinction, with a maximum at 640 nm. A similar increase is seen upon energization of internally facing cytochrome oxidase molecules in *c*-loaded COV. Both "passive" and "active" responses are only seen when the dye is fully bound to the vesicle membrane. Calibration curves using potassium or *n*-butyltriphenylphosphonium ion ($BTPP^+$) diffusion potentials are linear up to 100 mV and pass through the origin. Diffusion potentials (positive inside) also cause an increase and red shift in the oxonol V fluorescence emission spectrum. However, potentials of the same sign induced by cytochrome oxidase turnover induce a large fluorescence quenching in *c*-loaded COV. A similar anomaly has been observed with submitochondrial particles [Smith, J. C., Russ, P., Cooperman, B. S., & Chance, B. (1976) *Biochemistry 15*, 5094–5105]. A model is proposed consistent with these responses. It is suggested that the dye molecules move further into the membrane phase upon energization, causing the absorbance increase. In the presence of active enzyme, anionic dye molecules are attracted to a positive dipole on each enzyme molecule, causing self-quenching of the fluorescence.

Cytochrome *c* oxidase can be incorporated into proteoliposomes in either of two orientations (Nicholls et al., 1988). The membrane potential, $\Delta\Psi$ (negative inside the vesicle), developed during oxidase turnover on external cytochrome *c*, has been studied extensively. It has been measured directly as −100 mV by Drachev et al. (1976) in fusions of cytochrome oxidase proteoliposomes (COV)[1] and black lipid membranes. In standard COV preparations (as in mitochondria), indirect techniques must be adopted. Using a tetraphenylphosphonium ion (TPP^+) specific electrode, Casey et al. (1984) found a value of −115 mV and Vrij et al. (1986) determined a maximum of −100 mV. Similarly, Singh and Nicholls (1986) calculated a maximum $\Delta\Psi$ of −90 mV from results with a butyltriphenylphosphonium ($BTPP^+$) electrode. Much more negative values (varying between −150 and −170 mV) were reported by Singh and Nicholls (1985) using the fluorescent probes $diSC_3$-5 and safranine, such a larger value being independently confirmed by Miki and Orii (1986).

Cytochrome oxidase molecules may also be oriented in COV so that the cytochrome *c* binding sites face internally. Such enzyme species generate a membrane potential of the opposite sign (positive inside), as shown by uncoupler- and cyanide-sensitive accumulation of the permeant anion phenyldicarbaundecaborane (Jasaitis et al., 1972; Drachev et al., 1976). When cytochrome *c* loaded COV oxidize the membrane-permeable reductant TMPD, they accumulate the oxidation product Würster's Blue–$TMPD^+$ cation (Wrigglesworth & Nicholls, 1979), despite the presence of external ascorbate. The steady-state level of trapped $TMPD^+$ is dependent upon the presence of ionophores (Cooper & Nicholls, 1987; Nicholls et al., 1988) and also shows qualitatively the existence of a membrane potential (positive inside). But quantification requires a probe that is not directly affected by flux.

Fluorescence changes of the dye $diSC_3$-5 due to accumulation of the cationic form inside a vesicle may be used to monitor negative membrane potentials (Waggoner, 1976). To respond to a membrane potential, positive inside, a membrane-permeable anionic dye is needed. Absorbance changes in bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol (oxonol V) have been used to detect such positive membrane potentials. Smith et al. (1976) showed that it responds to passive diffusion potentials (+ve inside) as well as to active potentials in ATPase vesicles, in inverted submitochondrial particles [see also Chance et al. (1979)] and in *Rhodospirillum rubrum* chromatophores, all of which are positive inside. Measurements have also been made in bovine chromaffin granules (Scherman & Henry, 1980), Lettré cells (Bashford & Pasternak, 1984), whole neutrophils (Bashford & Pasternak, 1985), and cytoplasts from neutrophils (Henderson et al., 1987). The dye signal has been calibrated against potassium diffusion potentials (Bashford & Pasternak, 1984, 1985; Bashford et al., 1985; Henderson et al., 1987) and against proton diffusion potentials (Bashford & Pasternak, 1984; Bashford et al., 1985).

A related dye, oxonol VI, has also been used to measure membrane potentials in submitochondrial particles (Bashford & Thayer, 1977; Smith & Chance, 1979), *Rhodospirillum* chromatophores (Smith et al., 1978; Bashford et al., 1979a;

† Supported by Canadian Natural Sciences and Engineering Research Council Operating Grants A-0412 to P.N. and URF0043964 to D.B. and an Ontario Graduate Scholarship to C.E.C.

* To whom correspondence should be addressed.

‡ Present address: Section of Biochemistry, Division of Biomolecular Sciences, King's College, Campden Hill Rd., Kensington, London W8 7AH, U.K.

[1] Abbreviations: BTPP, *n*-butyltriphenylphosphonium; COV, cytochrome *c* oxidase proteoliposomes; *c*-loaded COV, COV containing entrapped cytochrome *c*; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; $diSC_3$-5, 3,3′-dipropylthiodicarbocyanine; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; oxonol V, bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TPP, tetraphenylphosphonium.

0006-2960/90/0429-3859$02.50/0

Armitage & Evans, 1981), Na^+/K^+ ATPase vesicles (Appel & Bersch, 1987), ATP synthetase vesicles (Van Walraven et al., 1985), and asolectin liposomes (Krab et al., 1985).

In the present paper we describe the use of oxonol V to measure the membrane potential in *c*-loaded COV. A mechanism of action consistent with the observed potential-sensitive absorbance and fluorescence responses of the dye is proposed.

Materials and Methods

Cytochrome *c* oxidase was purified from beef hearts according to the procedure of Kuboyama et al. (1972) with Tween 80 substituting for Emasol as the final detergent. Protein-free liposomes were prepared by the sonication technique. Dry asolectin was dispersed in buffer to a concentration of 50 mg/mL by mechanical mixing (leaving the asolectin in 50 mM sodium phosphate, pH 7.4, buffer at 4 °C overnight significantly reduces the amount of vortexing required without affecting the properties of the resulting liposomes). The suspension (5–10 mL) was then sonicated for 9–13 min in the pulsed mode at 30% duty cycle by using a Heat Systems-Ultrasonics Inc. W-375 sonicator. The solution was kept in an ice bath throughout to prevent overheating. Following sonication the liposomes were centrifuged at 29000*g* for 15 min, and the small pellet (consisting of titanium from the sonicator probe and large lipid aggregates) was discarded. The liposomes were then either used immediately or stored for up to a week at 4 °C. Cytochrome *c* containing cytochrome oxidase proteoliposomes were prepared by the addition of 100–125 μM cytochrome *c* (horse heart) and 3 μM cytochrome aa_3 to the asolectin suspension in 50 mM potassium phosphate, pH 7.4, prior to sonication, as described previously (Nicholls et al., 1980; Cooper & Nicholls, 1987). Excess external cytochrome *c* was removed by chromatography on Sephadex CM-25.

Absorption spectra were obtained on a Beckman DU-7 HS spectrophotometer interfaced to an Apple II Plus microcomputer as described by Chanady et al. (1985). Changes in oxonol V absorbance with time were measured with an Aminco DW-2 spectrophotometer operating in dual-beam mode using a thermostatically controlled cuvette holder. Cytochrome *a* absorbance changes rendered 612 nm (Bashford & Smith, 1979a) unsatisfactory as a reference wavelength. As the dye does not absorb light at 690 nm, either in passive or active dye/lipid interactions, the wavelength pair 640 minus 690 nm was used to follow dye/lipid binding *and* membrane energization. Samples were left in the cuvette until lipid binding was complete, before membrane potential formation was initiated. Dilution effects were corrected by subtracting the signal change induced by addition of an equal volume of water.

Oxonol V in aqueous media has an absorption maximum at 602 nm (Smith et al., 1976; Bashford et al., 1979b). Upon addition of phospholipid this peak is red-shifted to 625 nm as the dye enters a less polar environment (Smith et al., 1976; Bashford & Smith, 1979a; Bashford et al., 1979b). The change usually has a slow component; therefore, the dye was always left for at least 30 min after the vesicle addition until the spectrum had stabilized.

Room temperature fluorescence emission spectra were obtained as described in Bruce et al. (1989). A spectrofluorometer based on a Jarrell Ash $^1/_4$ meter spectrograph and an EG&G diode array detector (1420R) was controlled by an EG&G detector interface (1461) accessed by an IBM AT compatible computer. Excitation light (10-nm bandwidth) for the fluorescence emission was supplied by a 100-W tungsten halogen lamp dispersed by a Jobin Yvon H20 spectrometer.

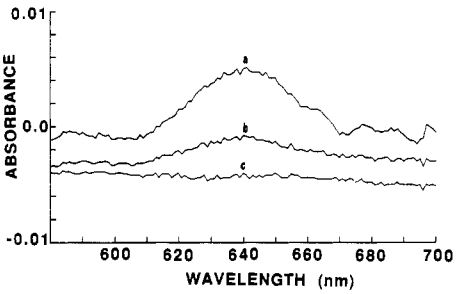

FIGURE 1: Effect of a diffusion potential (+ve inside) on the difference spectrum of oxonol V in vesicles. Oxonol V (5 μM) was added to sonicated liposomes (1.3 mg/mL phospholipid) in 50 mM sodium phosphate, pH 7.4 (internal and external buffer). Valinomycin (100 ng/mL) did not modify the spectrum. The spectrum of a valinomycin-treated sample was subtracted from the spectra following energization to yield the difference spectra shown: (a) +2 mM KCl (immediate spectrum); (b) after 20 s; (c) after 80 s.

The exposure time was 1 s; 5 scans were averaged per spectrum.

All experiments were carried out at 30 °C. Calculations of the equilibrium level of oxonol V binding to phospholipids were performed as described in Bashford et al. (1979b).

Asolectin (type IV-S, phosphatidylcholine), ascorbate (L-ascorbic acid, sodium salt), cytochrome *c* (horse heart, type VI), and valinomycin were all obtained from Sigma. BTPP (bromide) was obtained from the Aldrich, nigericin from Calbiochem, DAD from Polyscience, and oxonol V from Molecular Probes. FCCP was a kind gift of Dr. P. G. Heytler (Du Pont).

Results

Figure 1 shows that in the presence of the potassium ionophore, valinomycin, the addition of KCl to sonicated liposomes prepared in sodium phosphate causes an increase in absorbance with a maximum at 640 nm. The initial change (Figure 1a) decays with time (Figure 1b,c) in a manner similar to that of the fluorescent probe safranine responding to a diffusion potential (negative inside) in COV (Singh & Nicholls, 1985). The reversibility of the change shows that it is a response to the diffusion potential and not simply to the addition of potassium. The difference spectra in Figure 1 are analogous to the absolute spectra produced under similar conditions by Bashford and Smith (1979a). As the new peak is not associated with an absorbance loss elsewhere, it probably represents an increase in extinction of some dye, not a red shift of all of it. Some authors have suggested that oxonol response calibrations using K^+/valinomycin diffusion potentials are not accurate because valinomycin forms complexes with oxonols (Waggoner, 1976; Smith et al., 1978). Under the K^+-free conditions of Figure 1 there is, however, no evidence for such an interaction.

Cytochrome *c* loaded COV can be energized by a membrane-permeable reductant such as DAD [TMPD was not used as it forms Würster's Blue ($TMPD^+$) which interferes with the observed oxonol changes]. Figure 2 shows that the "active" potential during respiration (Figure 2a) induces the same spectral shift as the "passive" diffusion potential. The 640-nm peak was totally absent after anaerobiosis (Figure 2b) or in the presence of uncouplers (Figure 2c). As with the passive potentials, respiration-dependent energization caused only an absorbance increase; no troughs were seen.

The peak at 605 nm in parts a–c of Figure 2 is due to reduced cytochrome *a*, which is fully reduced after anaerobiosis (Figure 2b). Addition of uncouplers increased the steady-state reduction of cytochrome *a* as in controlled "right-side-out"

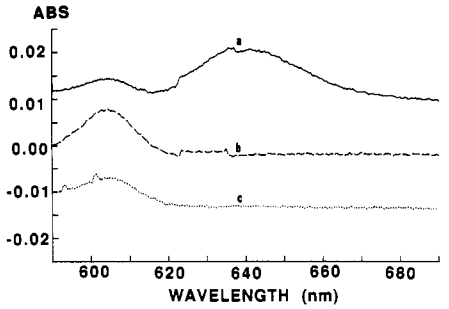

FIGURE 2: Effect of internal turnover in *c*-loaded COV on the difference spectrum of oxonol V. Oxonol V (3.6 μM) was added to *c*-loaded COV (0.33 μM aa_3 and 3.3 mg/mL phospholipid) in 50 mM potassium phosphate, pH 7.4 (internal and external buffer). Addition of 1 mM sodium ascorbate alone had no effect on the spectrum. The spectrum of the ascorbate-treated sample was subtracted from that obtained upon energization to produce the difference spectra shown: (a) +75 μM DAD (after 1 min); (b) after 6 min (anaerobic); (c) as (a), plus 5 μg/mL valinomycin and 5 μM FCCP.

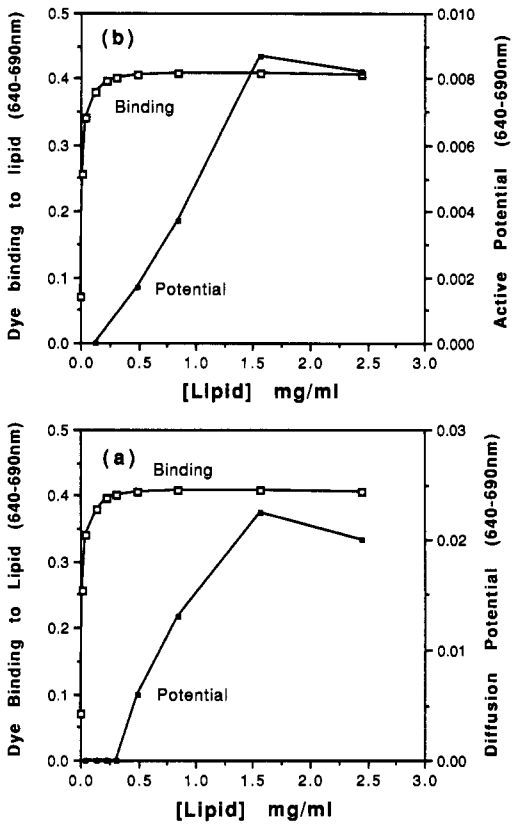

FIGURE 3: Effect of vesicle concentration on the spectral response of oxonol V to active and passive potentials. (a) Protein-free liposomes. Extent of dye binding (□) and potential (■) were monitored at 640–690 nm as a function of vesicle concentration. Dye binding was initiated by the addition of sonicated liposomes (internal buffer sodium phosphate, pH 7.4) to 5 μM oxonol V in 50 mM sodium phosphate, pH 7.4, plus 100 ng/mL valinomycin. After binding was complete, 2 mM KCl was added and the signal allowed to stabilize. A diffusion potential of 62 mV was then imposed across the liposomal membrane by addition of 20 mM KCl. (b) Cytochrome oxidase proteoliposomes. As in (a) except that the medium was 50 mM potassium phosphate, pH 7.4, in the absence of valinomycin and *c*-loaded COV (internal buffer 50 mM potassium phosphate, pH 7.4) were used instead of liposomes. The active potentials were initiated by the addition of 5 mM sodium ascorbate and 0.5 mM DAD.

COV (Wrigglesworth & Nicholls, 1978; Gregory & Ferguson-Miller, 1989). Thus, respiration in *c*-loaded COV is responsive to the proton motive force [cf. Cooper and Nicholls (1987, 1990)].

The changes in Figures 1 and 2 were observed under conditions where over 98% of the dye was initially bound to lipid.

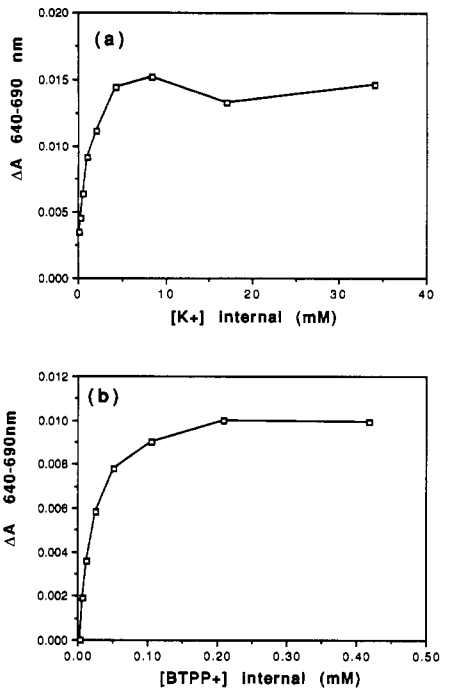

FIGURE 4: Effect of internal cation concentration on the oxonol V resonse to diffusion potential. (a) Potassium–valinomycin system. Oxonol V (10 μM) was bound to 3.3 mg/mL sonicated liposomes (internal buffer 50 mM sodium phosphate, pH 7.4). External buffer was 50 mM sodium phosphate, pH 7.4, with 100 ng/mL valinomycin and 0.13 mM KCl. External and internal solutions were allowed to equilibrate. Successive KCl pulses were then added to impose a membrane potential at 18.6 mV (+ve inside) at different internal [K^+]. The oxonol V response was monitored at 640–690 nm. (b) BTPP system. As in (a), except that 5 μM oxonol V was bound to 1.65 mg/mL liposomes and additions of $BTPP^+Br^-$ (in the absence of valinomycin) were made instead of KCl.

As these changes apparently involve fully bound dye, it probably does not function under these conditions as a simple redistribution probe between aqueous and lipid phases, as postulated by Smith et al. (1976) and by Scherman and Henry (1980) for their rather different systems. In this respect it is unlike $diSC_3$-5 (Waggoner, 1979) and possibly also unlike oxonol VI (Appel & Bersch, 1987). The red shift of the oxonol V spectrum is a probable consequence of a transfer to a more nonpolar, possibly amphipathic, environment (Smith et al., 1976; Bashford et al., 1979b), but not into the aqueous phase.

The effect of lipid concentration on the probe response therefore required study to provide mechanistic information and also to optimize the dye/lipid ratios needed for quantitation of the membrane potential measurement [cf. Singh and Nicholls (1985)]. Figure 3 shows the effect of lipid concentration on the dye response to either a diffusion (Figure 3a) or an active potential (Figure 3b). At 5 μM oxonol V, the two types of potential induce similar responses; each increases the 640–690-nm signal to a maximum value at 1.5 mg/mL lipid (when 99% of the dye is membrane-bound). No appreciable signal can be seen until the dye is almost completely bound (at 0.5 mg/mL lipid, 96% has combined). A significant increase in absorbance is then seen over a very small range of lipid concentration. As in Figures 1 and 2 the dye is present entirely in the lipid phase when the maximum effect is shown. Figure 3 even suggests that free dye may inhibit the energization response of bound dye.

Irreversible absorbance changes in oxonol V sometimes occur upon transfer from Na^+ to K^+ solutions (Cooper, 1989). The dependence of the reversible potential-sensitive signal on interal K^+ concentration was therefore also determined. Figure

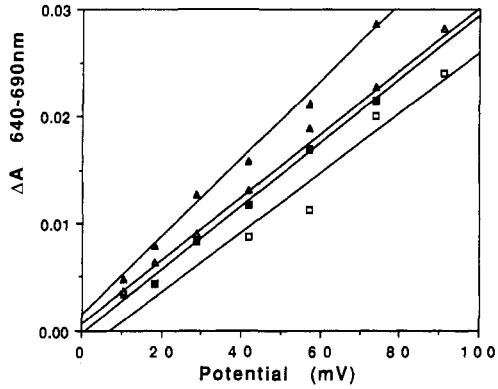

FIGURE 5: Calibration of oxonol V response to membrane potential. Oxonol V (5 μM) was bound to 1.7 mg/mL phospholipid. Diffusion potentials were imposed by addition of varying [KCl] or [$BTTP^+$ Br^-]. Absorbance change at 640–690 nm (corrected for dilution effects) is plotted against imposed potential (calculated as a Nernst potential). (□, ■) Potassium diffusion potentials; (□) sonicated liposomes in 50 mM sodium phosphate, pH 7.4, 1 mM KCl, 100 ng/mL valinomycin; (■) as above, but [KCl] = 2 mM. (△, ▲) $BTTP^+$ diffusion potentials; (▲) sonicated liposomes in 50 mM sodium phosphate, pH 7.4, with 50 μM $BTTP^+Br^-$; (△) *c*-loaded COV in 50 mM potassium phosphate, pH 7.4, with 50 μM $BTTP^+Br^-$.

4a shows that the oxonol response to a +18.6-mV potential increases to a maximum at about 10 mM internal [K^+]. This suggests that a K^+/oxonol V complex may be required for the dye response. However, K^+ effects on the oxonol absorbance are seen at cation concentrations below 2 mM (Cooper, 1989). An alternative way to measure the potential is to use the permeable cation $BTPP^+$. Figure 4b shows the result of such an experiment; a maximum signal is achieved at only 0.2 mM $BTPP^+$. Therefore, a specific K^+/oxonol complex is unlikely to be responsible for the behavior seen in Figure 4a.

The maximal effects (Figure 4) occur at quite high K^+ and $BTPP^+$ concentrations. Standard curves were constructed by using internal K^+ concentrations of 1 and 2 mM and an internal $BTPP^+$ concentration of 50 μM, which allowed potentials up to 100 mV to be measured. The results are given in Figure 5; each plot is linear, although least-squares linear regression lines do not all pass through the origin. Figure 5 also shows that $BTPP^+$-induced diffusion potentials, obtained in the (Na-containing) vesicles used for the K^+ calibrations, give a small *Y*-axis intercept, perhaps due to an absence of interaction between K^+ and oxonol V; in the presence of potassium, the $BTPP^+$ plot passed through or near to the origin. $BTPP^+$ has the advantage that calibrations can be performed in the same vesicles that are used for enzyme-induced energization. Measurements of $\Delta\Psi$ in *c*-loaded COV using the $BTPP^+$ calibration give a maximum potential of +53 mV (Cooper & Nicholls, 1990).

The fluorescence of oxonol V is also sensitive to energization and has been used as a probe in a number of systems (Smith et al., 1976, 1978, 1980, 1981; Scherman & Henry, 1980). Unlike the absorbance changes, fluorescence responses to active potentials often seem not to correlate with those due to passive diffusion potentials (Smith et al., 1976). We have, nevertheless, examined the fluorescence changes of oxonol V in the cytochrome *c* loaded cytochrome oxidase containing vesicles.

The fluorescence emission spectrum of oxonol V, like the absorbance spectrum, is red-shifted upon binding to lipid membranes (Smith et al., 1978; Scherman & Henry, 1980). Figure 6 shows that oxonol V is sonicated liposomes fluoresces with an emission maximum at 655 nm, similar to that seen in submitochondrial particles (Smith et al., 1978) and in bovine chromaffin granules (Scherman & Henry, 1980). A K^+

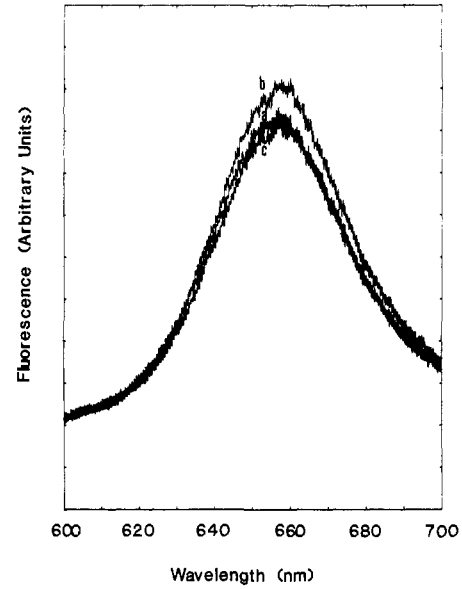

FIGURE 6: Effect of a diffusion potential (+ve inside) on the fluorescence emission spectrum of oxonol V in vesicles. Fluorescence emission spectra were recorded as described under Materials and Methods with an excitation wavelength of 580 nm. Oxonol V (5 μM) was bound to sonicated liposomes (1.3 mg/mL phospholipid) in 50 mM sodium phosphate, pH 7.4 (internal and external buffer), plus 100 ng/mL valinomycin. (a) Control (equilibrium state); (b) plus 2 mM KCl (immediate); (c) after 5 min.

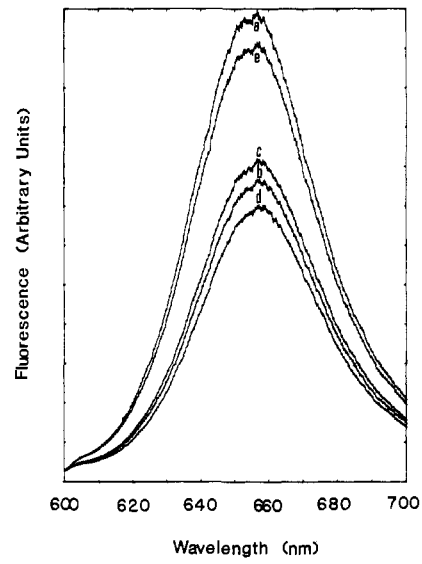

FIGURE 7: Effect of nigericin and valinomycin on the fluorescence emission spectrum of oxonol V following energization in *c*-loaded COV. Fluorescence emission spectra were obtained as in Figure 6. Oxonol V (5 μM) bound to *c*-loaded COV (43 nM aa_3 and 1.3 mg/mL phospholipid) in 50 mM potassium phosphate, pH 7.4 (internal and external buffer), with 5 mM sodium ascorbate. (a) Control (at equilibrium); (b) plus 125 μM DAD (immediate); (c) after 5 min (steady state); (d) plus 100 ng/mL nigericin; (e) plus 100 ng/mL valinomycin.

diffusion potential (positive inside), increases the fluorescence, and there is a slight red shift. The increase in reversible with time as the diffusion potential collapses, as are the absorbance changes observed under identical conditions (Figure 1).

However, in contrast to the similarities of the active and passive absorbance changes (cf. Figures 1 and 2), Figure 7 shows a dramatic difference between the fluorescence responses to active and passive membrane potentials. A large fluorescence decrease is observed upon initiation of internal turnover in *c*-loaded COV (compare traces a and b in Figure 7). The

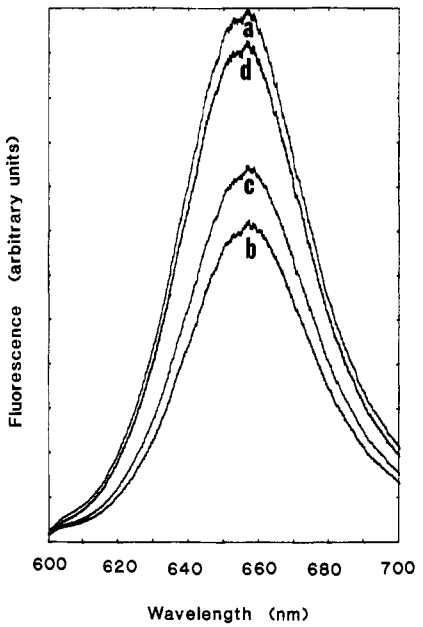

FIGURE 8: Effect of anaerobiosis on the oxonol V fluorescence changes associated with internal turnover in *c*-loaded COV. Fluorescence emission spectra were obtained as in Figure 6. Oxonol V (5 μM) was bound to *c*-loaded COV (43 nM aa_3 and 1.3 mg/mL phospholipid) in 50 mM potassium phosphate, pH 7.4 (internal and external buffer). (a) Control (equilibrium); (b) plus 5 mM sodium ascorbate and 125 μM DAD (immediate); (c) after 10 min (steady state); (d) after 20 min (anaerobic).

response, although opposite in sign to that induced passively, tracks the changing membrane potential. Thus, the steady-state "quench" decreases with time as $\Delta\Psi$ converts to ΔpH (Figure 7c). Nigericin collapses ΔpH, and the fluorescence quench increases as $\Delta\Psi$ is restored, reaching a maximal value (Figure 7d). If $\Delta\Psi$ is abolished with valinomycin (Figure 7e), the preenergization fluorescence is fully restored (apart from a small dilution artifact). This fluorescence quenching behavior, which is unaccompanied by any shift in peak position, is similar to that obtained when submitochondrial particles (Smith et al., 1978, 1981) or bovine chromaffin granules are energized with ATP (Scherman & Henry, 1980).

Figure 8 shows that if uncoupler is not present, the extent of fluorescence quenching after addition of DAD decreases with time until it reaches a steady-state value. Upon anaerobiosis the fluorescence is restored to its initial level.

Discussion

As the potential-induced shift in the oxonol V absorbance is only seen when the dye is almost fully bound to the membrane, the mechanism of oxonol V action under our conditions cannot be a simple redistribution between the aqueous and membrane phases [contrast Smith et al. (1976) and Scherman and Henry (1980)]. It is possible that it acts electrochromically (like carotenoids) and that the red shift is a consequence of an interaction of the electrical field across the membrane with the dipole moments of the dye in the ground and excited states (Smith et al., 1976). However, Scherman and Henry (1980) claim that the response time of the dye is too slow for such an effect. They also found no evidence in chromaffin granules that the energized spectrum was different from the bound spectrum (in contrast to Figures 1 and 2) and therefore postulated a mechanism wherein energization increased the number of binding sites available to the dye. Under other conditions (Schuurmans et al., 1978; Peters et al., 1983) fast responses are seen with oxonol VI (in our experiments the responses were complete within the mixing times involved). If energization allows some molecules to bind to more polar sites in the membrane, thus red shifting their spectra and increasing absorbances, the two sets of findings may be reconcilable. Smith and co-workers (Smith et al., 1978, 1981) propose that oxonol normally occupies a site near the hydrophobic portion of the bilayer—with the two phenyl side chains extending into the interior. Waggoner et al. (1977) and Smith et al. (1980) suggest that energization triggers a rapid transfer of dye molecules from an aqueous or hydrophilic region near the surface of the membrane to a hydrophobic binding site in the bilayer. The energization-dependent sites are available deeper in the bilayer.

The response of oxonol V to diffusion potentials is dependent upon internal cation concentration (cf. Figure 4). Krab et al. (1985) showed a specific threshold for the response of oxonol VI to K^+/valinomycin-induced diffusion potentials in sonicated liposomes. Below the threshold no signal is seen, but above it the response is Nernstian. They claim that this indicates a resting potential (positive outside), which the diffusion potential must overcome before it can trigger an oxonol response. With increasing internal [K^+], the threshold is reduced, and so increasing [K^+] or [$BTPP^+$], will increase the response at a given diffusion potential, as seen in Figure 4.

Although plots of imposed diffusion potential against oxonol V response (Figure 5) show a dependence on internal [K^+] similar to that found by Krab et al. (1985), for oxonol VI the corresponding intercepts were closer to the origin. Krab et al.'s (1985) sonication method may have achieved a homogeneous population of very small vesicles; with proteoliposomes, extended sonication inhibits enzyme activity (Wrigglesworth, 1988). The smaller the vesicle, the further from the origin is the intercept (Krab et al., 1985).

Oxonol V absorbance responses with passive gradients are linear over a range of potentials (0–100 mV), and the calibrations can be made in the same *c*-loaded COV used for respiration-induced turnover. The oxonol V method therefore compares favorably with the methods for "normal" COV ($\Delta\Psi$ negative inside) using the probes $diSC_3$-5 and safranine. Their calibration curves also fail to pass through the origin and are highly nonlinear at low dye/lipid ratios (Singh & Nicholls, 1985). However, they can be used up to nearly −200 mV, which would be difficult with oxonol V; at the very low internal [K^+] needed the signal would be small.

In response to a passive $\Delta\Psi$ across the liposomal membrane, the absolute oxonol V fluorescence changes are similar to those seen in the absorbance spectrum [cf. Bashford and Smith (1979a) and Figure 1]. As the diffusion potential causes little absorbance change at the excitation wavelength (580 nm, cf. Figure 1), the fluorescence effect is probably a direct action of $\Delta\Psi$ on the emission spectrum. To explain the large fluorescence *decrease* observed when an *active* $\Delta\Psi$ of the same sign is imposed, Smith et al. (1976, 1981) proposed that upon energization the probe redistributes from the aqueous phase into the membrane, with consequent self-quenching. Oxonol V fluorescence intensity in ethanol is indeed nonlinear above a concentration of 2 μM (Smith et al., 1976). However, a simple aqueous/membrane shift fails to explain why the diffusion and enzyme-linked potentials have different effects; oxonol V fluorescence must respond in some way to the energy-transducing enzymes themselves.

If the oxidase created a high "local" potential, this might explain the fluorescence anomaly. The existence of such potentials has been postulated but controversial for many years (Ferguson, 1985), and it is not clear how they could exist in a proteoliposome. However, any energy-transducing enzyme

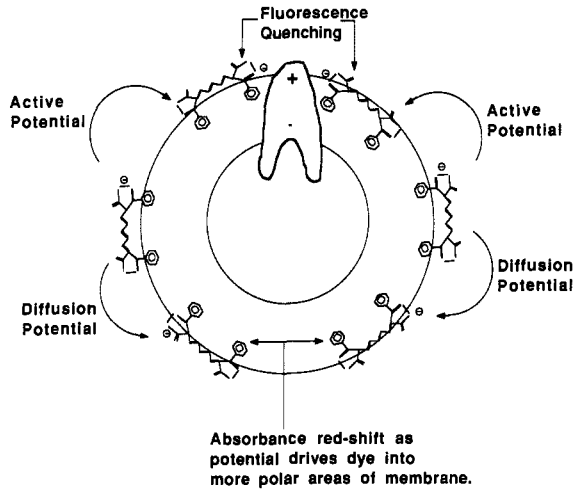

FIGURE 9: Model of the absorbance and fluorescence changes associated with the response of oxonol V to active and passive potentials across the proteoliposomal membrane.

that separates charge across a lipid bilayer must have a strong dipole (Westerhoff et al. (1987). If following energization oxonol moves further into the hydrophobic phase of the membrane, as proposed by Smith and Chance (1979) for submitochondrial particles, and also intimated by Bashford et al. (1979b) for asolectin bilayers, it may come under the influence of the oxidase dipoles. Negative dye molecules attracted to the positive end of the dipole would represent an increased concentration of dye and show self-quenching. The absorbance increases linearly with dye concentration up to very high values (Smith et al., 1976; Cooper 1989); the only effect on the spectrum is that of the nonpolar shift. The absorbance responses are therfore identical in the presence and absence of oxidase, but the fluorescence responses are not. The proposed model is illustrated in Figure 9. Whether or not this model has any resemblance to reality, the fact that oxonol V fluorescence in COV responds in an opposite fashion to diffusion and to active potentials, whereas the absorbance changes in the two cases are similar, is in agreement with results obtained by using submitochondrial particles (Smith et al., 1976). The *c*-loaded COV thus mimic SMP in this, as in other, respects (Cooper & Nicholls, 1990), even though it is as yet not possible to quantify the oxonol V fluorescence behavior.

REFERENCES

Appel, H.-J., & Bersch, B. (1987) *Biochim. Biophys. Acta 903*, 480–494.

Armitage, J. P., & Evans, M. C. W. (1981) *FEBS Lett. 126*, 98–102.

Bashford, C. L., & Thayer, W. S. (1977) *J. Biol. Chem. 252*, 8459–8463.

Bashford, C. L., & Smith, J. C. (1979a) *Methods Enzymol. 55*, 569–586.

Bashford, C. L., & Smith, J. C. (1979b) *Biophys. J. 25*, 81–85.

Bashford, C. L., & Pasternak, C. A. (1984) *J. Membr. Biol. 79*, 275–284.

Bashford, C. L., & Pasternak, C. A. (1985) *Biochim. Biophys. Acta 817*, 174–180.

Bashford, C. L., Chance, B., & Prince, R. C. (1979a) *Biochim. Biophys. Acta 545*, 46–57.

Bashford, C. L., Chance, B., Smith, J. C., & Yoshida, T. (1979b) *Biophys. J. 25*, 63–80.

Bashford, C. L., Alder, G. M., Gray, M. A., Micklem, K. J., Taylor, C. C., Turek, P. J., & Pasternak, C. A. (1985) *J. Cell. Physiol. 123*, 326–336.

Bruce, D., Brimble, S., & Bryant, D. A. (1989) *Biochim. Biophys. Acta 974*, 66–73.

Casey, R. P., O'Shea, P. S., Chappell, J. B., & Azzi, A. (1984) *Biochim. Biophys. Acta 765*, 30–37.

Chanady, G. A., Chang, E., & Proteau, G. (1985) *Biochem. Cell. Biol. 63*, 160–161 (Appendix to paper by Sone and Nicholls).

Chance, B., Nakase, Y., & Itshak, F. (1979) *Arch. Biochem. Biophys. 198*, 360–369.

Cooper, C. E. (1989) Ph.D. Thesis, University of Guelph, ON, Canada.

Cooper, C. E., & Nicholls, P. (1987) *FEBS Lett. 223*, 155–160.

Cooper, C. E., & Nicholls, P. (1990) *Biochemistry* (following paper in this issue).

De Vrij, W., Dreissen, A. J. M., Hellingwerf, K. J., & Konings, W. N. (1986) *Eur. J. Biochem. 156*, 431–440.

Drachev, L. A., Jasaitis, A. A., Kaulen, A. D., Kondrashin, A. A., Chu, L. V., Semenov, A. Y., Severina, I. I., & Skulachev, V. P. (1976) *J. Biol. Chem. 251*, 7072–7076.

Ferguson, S. J. (1985) *Biochim. Biophys. Acta 811*, 47–95.

Gregory, L., & Ferguson-Miller, S. (1989) *Biochemistry 28*, 2655–2662.

Henderson, L., Chappell, J. B., & Jones, O. T. G. (1987) *Biochem. J. 246*, 325–329.

Jasaitis, A. A., Nemecek, I. B., Skulachev, V. P., & Smirnova, S. M. (1972) *Biochim. Biophys. Acta 275*, 485–490.

Krab, K., Van Walraven, H. S., Scholts, M. J. C., & Kraayenhof, R. (1985) *Biochim. Biophys. Acta 809*, 228–235.

Kuboyama, M., Yong, F. C., & King, T. E. (1972) *J. Biol. Chem. 247*, 6375–6383.

Miki, T., & Orii, Y. (1986) *J. Biochem. 100*, 735–745.

Nicholls, P., Hildebrandt, V., & Wrigglesworth, J. M. (1980) *Arch. Biochem. Biophys. 204*, 533–543.

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.

Peters, F. A. L. J., Van Spanning, R., & Kraayenhof, R. (1983) *Biochim. Biophys. Acta 724*, 159–165.

Scherman, D., & Henry, J. P. (1980) *Biochim. Biophys. Acta 559*, 150–156.

Schuurmans, J. J., Casey, R. P., & Kraayenhof, R. (1978) *FEBS Lett. 94*, 405–409.

Singh, A. P., & Nicholls, P. (1985) *J. Biochem. Biophys. Methods. 11*, 95–108.

Singh, A. P., & Nicholls, P. (1986) *Arch. Biochem. Biophys. 245*, 436–445.

Smith, J. C., & Chance, B. (1979) *J. Membr. Biol. 46*, 255–282.

Smith, J. C., Russ, P., Cooperman, B. S., & Chance, B. (1976) *Biochemistry 15*, 5094–5105.

Smith, J., Powers, L., Prince, R., Chance, B., Bashford, L. (1978) in *Frontiers of Biological Energetics* (Scarpa, A., Dutton, P. L., & Leigh, J. S., Eds.) Vol. 2, pp 1293–1303, Academic Press, New York.

Smith, J. C., Frank, S. J., Bashford, C. L., Chance, B., & Rudkin, B. (1980) *J. Membr. Biol. 54*, 127–139.

Smith, J. C., Hallidy, L., & Topp, M. R. (1981) *J. Membr. Biol. 60*, 173–185.

Van Walraven, H. S., Hagendoorn, M. J. M., Krab, K., Haak, N. P., & Kraayenhof, R. (1985) *Biochim. Biophys. Acta 809*, 236–244.

Waggoner, A. (1976) *J. Membr. Biol. 27*, 317–334.
Waggoner, A. (1979) *Annu. Rev. Biophys. Bioenerg. 8*, 47–68.
Waggoner, A. S., Wang, C. H., & Tolles, R. L. (1977) *J. Membr. Biol. 33*, 109–140.
Westerhoff, H. V., Kamp, F., Tsong, T. Y., & Astumian, R. D. (1987) in *Mechanistic approaches to interactions of electric and electromagnetic fields with living systems* (Blank, M., & Findl, E., Eds.) pp 203–215, Plenum Press, New York.
Wrigglesworth, J. M. (1988) *Mol. Aspects Med. 10*, 223–232.
Wrigglesworth, J. M., & Nicholls, P. (1978) *FEBS Lett. 91*, 190–193.
Wrigglesworth, J. M., & Nicholls, P. (1979) *Biochim. Biophys. Acta 547*, 36–46.

Structure and Vectorial Properties of Proteoliposomes Containing Cytochrome Oxidase in the Submitochondrial Orientation†

Chris E. Cooper‡ and Peter Nicholls*
Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada L2S 3A1
Received September 14, 1989; Revised Manuscript Received December 18, 1989

ABSTRACT: Cytochrome oxidase proteoliposomes were prepared from bovine heart oxidase. Size distributions determined by quasi-elastic light scattering (QELS) showed that there was a small population of large vesicles (120–200-nm diameter) and a large population of small vesicles (50–100-nm diameter). Trapping cytochrome *c* inside the proteoliposomes did not significantly alter this size distribution. Separation of the vesicles by gel filtration, however, revealed that the cytochrome *c*/cytochrome *a* ratio is higher in the larger vesicles. Internally trapped cytochrome *c* can be reduced by the membrane-permeable reductants 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) or *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD). Respiration on internal cytochrome *c* generated a membrane potential of 53 mV (positive inside) and a pH gradient of 0.2 (acid inside) as monitored by the optical probes oxonol V and pyranine, respectively. But the true magnitude of these gradients in individual proteoliposomes is complicated by vesicle heterogeneity. The membrane potential increased biphasically with increasing concentration of reductant. Ionophore sensitivity was higher for the "low K_m" phase, and respiration became increasingly uncoupled as the reductant concentration was increased. These findings are consistent with a kinetic heterogeneity such that vesicles respiring at lower reductant concentrations generate a higher proton motive force than those with a larger K_m. The steady-state internal acidification induced by turnover of the internally facing enzyme is probably maintained by both cytochrome oxidase proton translocation and a $TMPD^+/H^+$ antiport present in these vesicles [Cooper, C. E., & Nicholls, P. (1987) *FEBS. Lett. 223*, 155–160].

Cytochrome *c* oxidase vesicles capable of respiring on internally trapped cytochrome *c* (*c*-loaded COV)[1] can be prepared simply by the preaddition of cytochrome *c* to the reconstitution medium (Racker & Kandrach, 1971; Nicholls et al., 1980; Cooper & Nicholls, 1987). The internally facing enzyme in such *c*-loaded vesicles turns over upon the addition of membrane-permeable reductants such as phenazine methosulfate (Racker & Kandrach, 1971) or TMPD (Cooper & Nicholls, 1987). An apparently low turnover number for the enzyme respiring on internal cytochrome *c* is due to a high K_m for TMPD (Cooper & Nicholls, 1987). In the aerobic steady state, cytochrome *c* loaded COV accumulate $TMPD^+$ (Würster's Blue); the effects of ionophores on the $TMPD^+$ level provide indirect evidence that cytochrome oxidase respiring on internal cytochrome *c* generates a $\Delta\mu H^+$ of an orientation opposite to that generated by enzyme respiring on external cytochrome *c* (Cooper & Nicholls, 1987; Nicholls et al., 1988a).

However, the response of internally facing enzyme to ionophores is poor (Nicholls et al., 1980; Cooper & Nicholls, 1987), and some authors have suggested that internally facing enzyme molecules do not incorporate successfully into proteoliposomes (Madden et al., 1987). We therefore decided to analyze the structure of *c*-loaded COV to determine whether addition of cytochrome *c* causes any morphological changes to the vesicles; we have also used $\Delta\mu H^+$-sensitive probes to measure directly the proton motive force that is generated by the oxidation of internal cytochrome *c*. The results show that cytochrome *c* loaded COV are structurally indistinguishable from standard COV but that they generate a significant proton motive force of an orientation opposite to that generated in such standard COV.

Materials and Methods

Cytochrome *c* oxidase was purified from beef hearts according to the procedure of Kuboyama et al. (1972) with Tween 80 substituting for Emasol as the final detergent. Liposomes and cytochrome oxidase proteoliposomes (COV) were prepared by the sonication technique as described previously (Proteau et al., 1983). Cytochrome *c* loaded COV were also made by sonication, and the excess, external, cytochrome

† Supported by Canadian Natural Sciences and Engineering Research Council Operating Grant A-0412 to P.N. and an Ontario Graduate Scholarship to C.E.C.
* To whom correspondence should be addressed.
‡ Present address: Biochemistry Section, Division of Biomolecular Sciences, King's College, Campden Hill Rd., Kensington, London W8 7AH, U.K.

[1] Abbreviations: BTPP, *n*-butyltriphenylphosphonium; COV, cytochrome *c* oxidase proteoliposomes; *c*-loaded COV, COV containing entrapped cytochrome *c*; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; $diSC_3$-5, 3,3′-dipropylthiodicarbocyanine; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; oxonol V, bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol; pyranine, trisodium 8-hydroxy-1,3,6-pyrenetrisulfonate; QELS, quasi-elastic light scattering; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

0006-2960/90/0429-3865$02.50/0