Steady-state kinetics of ubiquinol–cytochrome *c* reductase in bovine heart submitochondrial particles: diffusional effects

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In an attempt to establish the relative importance of diffusional and chemical control in the reactivity of the two substrates, ubiquinol and cytochrome *c*, we have undertaken an extensive characterization of the steady-state kinetics of ubiquinol–cytochrome *c* reductase (EC 1.10.2.2) when present in open submitochondrial particles from bovine heart. The kinetic pattern follows a Ping Pong mechanism; contrary to the situation found with the isolated enzyme [Speck and Margoliash (1984) *J. Biol. Chem.* **259**, 1064–1072, and confirmed in our laboratory], no substrate inhibition by oxidized cytochrome *c* was observed with the membrane-bound enzyme. Endogenous oxidized ubiquinone-10 is unable to exert product inhibition under the conditions employed. In the Ping Pong mechanism for this enzyme, the reaction scheme can be clearly divided into two parts, and the $K_{\text{min.}}$ ($k_{\text{cat.}}/K_m$) value for one substrate is independent of the rate constant for the second substrate. Both ubiquinol-1 and ubiquinol-2 can be used as electron donors reacting with the enzyme from within the lipid bilayer [Fato, Castelluccio, Palmer and Lenaz (1988) *Biochim. Biophys. Acta* **932**, 216–222]; the $k_{\text{min.}}$ values for ubiquinols, when calculated on the basis of their membranous concentrations, are significantly lower than the $k_{\text{min.}}$ for cytochrome *c*. The temperature-dependence of the kinetic parameters was investigated by titrating each of the substrates under quasi-saturating concentrations of the second substrate. Arrhenius plots of $V_{\text{max.}}$ extrapolated from both

cytochrome *c* and ubiquinol titrations were linear, when care was taken to verify the quasi-saturating concentrations of the fixed co-substrate. The Arrhenius plots for the $k_{\text{min.}}$ values for both ubiquinol and cytochrome *c* were linear, but the activation energy was much higher for the former, particularly when calculated for ubiquinol dissolved in the lipid phase; the very low value of activation energy of the $k_{\text{min.}}$ for cytochrome *c* is strong support for diffusion control being present in the reaction of cytochrome *c* with the membranous enzyme. In contrast to the soluble enzyme, ubiquinone titrations of submitochondrial particles at low cytochrome *c* concentrations deviated from hyperbolic behaviour. Changing the medium viscosity with either poly(ethylene glycol) or sucrose had a strong effect on the cytochrome *c* $k_{\text{min.}}$, whereas the change in the ubiquinol $k_{\text{min.}}$ was much smaller. From the viscosity studies the extent of diffusional control could be calculated, revealing that the reaction with cytochrome *c* was mostly diffusion-limited. The viscosity of the membrane was changed by incorporating cholesterol; no significant effect on the ubiquinol $k_{\text{min.}}$ ascribable to diffusion control could be recognized. The bulk of the studies reported here strongly suggest that ubiquinol–cytochrome *c* reductase is subject to diffusion control of the interaction of cytochrome *c* from the aqueous medium, but not of the interaction of the short-chain ubiquinols from the membrane interior.

INTRODUCTION

Several enzymic reactions occurring in membranes proceed at high rates, suggesting that they are diffusion-limited. In fact, high ratios of $k_{\text{cat.}}/K_m$, a parameter equated to the minimum value of the association rate constant ($k_{\text{min.}}$) in the classical Michaelis–Menten scheme, are suggestive of a diffusion-controlled rate process (Hasinoff, 1982). Diffusion-limited reactions are strongly sensitive to the viscosity of the medium (Berg and Von Hippel, 1985), an additional reason why reactions occurring in membranes, whose viscosity is higher than that of water (McCloskey and Poo, 1985), might be subject to diffusional control.

To establish the relevance of diffusion-controlled reactions in membrane-bound enzymes, we have chosen for investigation the ubiquinol–ferricytochrome *c* oxidoreductase (EC 1.10.2.2) (Rieske and Ho, 1985), as this membrane-bound enzyme has a catalytic-centre activity that is amongst the highest so far reported (Lenaz et al., 1985). In eukaryotic cells, the enzyme is situated in the mitochondrial inner membrane, where it constitutes the so-

called cytochrome *bc*₁ complex, or complex III (Rieske and Ho, 1985). It catalyses the redox reaction:

where cyt.*c*(ox.) and cyt.*c*(red.) are oxidized and reduced cytochrome *c* respectively, and QH₂ is the reduced ubiquinone (ubiquinol); it is usually ubiquinol-10 in mammalian mitochondria (Ramadasarma, 1968).

An understanding of the kinetics of this enzyme is complicated by the presence of at least two, and possibly three, distinct catalytic sites, one for ubiquinol oxidation, a second for cytochrome *c* reduction (Speck and Margoliash, 1984) and a postulated third reactive site (the so-called centre i) at which oxidized ubiquinone may be reduced by a *b* cytochrome in a reversible reaction (Berry and Trumpower, 1985). In principle, a diffusion-limited step could be present with either or both substrates; nevertheless, while cytochrome *c* reacts with the enzyme from the aqueous environment bathing the membrane outer surface (Hackenbrock et al., 1986), the very hydrophobic ubiquinol-10

Abbreviations used: 12-AS, 12-(9-anthroxyl)stearic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; $k_{\text{min.}}$, the minimal association rate constant of enzyme to substrate, corresponding to the $k_{\text{cat.}}/K_m$ ratio; PEG, poly(ethylene glycol); SMP, submitochondrial particles.

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reacts with the enzyme from within the lipid phase (Fato et al., 1988b). Thus while diffusional control would involve the physical properties (the fluidity) of the membrane phase for ubiquinol, it would depend primarily on the properties of the aqueous medium and/or of the membrane surface in the case of cytochrome *c* (Hackenbrock et al., 1986).

Additional problems are imposed by the hydrophobic nature of the electron donor, ubiquinol. The kinetics of the isolated enzyme have been investigated utilizing as reducing substrates either physiological water-insoluble long-chain ubiquinols in the presence of non-ionic detergents (Weiss and Wingfield, 1979; Engel et al., 1980), or water-soluble short-chain ubiquinols in the absence of detergents (Rieske, 1967; Speck et al., 1979; Degli Esposti and Lenaz, 1982a; Kauten et al., 1987). Only short-chain ubiquinols, however, can be used as donors to the enzyme in intact mitochondrial membranes, as detergents cannot be utilized with these organelles. Thus the oxidation site for ubiquinol can be easily reached by quinols dissolved in the aqueous medium, and the presence of endogenous ubiquinone-10 does not appear to be necessary for oxidation of exogenous short-chain ubiquinols other than duroquinol (Degli Esposti and Lenaz, 1982a; Zhu et al., 1982).

The presence of a diffusion-limited step in ubiquinol-cytochrome *c* reductase action has important physiological implications. The natural enzyme oxidizes ubiquinone that has been reduced by any of a series of flavin dehydrogenases, via the pool behaviour first analysed by Kröger and Klingenberg (1973) and later by Ragan and co-workers (Ragan and Cottingham, 1985). This analysis reveals that the overall observed rate of electron transfer is a hyperbolic function of the rates by which ubiquinone is reduced and oxidized, elaborated with a simple mathematical expression.

Hackenbrock et al. (1986) have proposed a random collision model for the mitochondrial respiratory chain, where the respiratory complexes are randomly dispersed in the lipid bilayer and electron transfer occurs by collisional encounters between the complexes and smaller connecting molecules, such as ubiquinone and cytochrome *c*. In this model, the collisional frequencies are comparable with the catalytic-centre activities of the respiratory chain, and the overall respiratory activity is explicitly postulated to be controlled by the lateral diffusion of the fastest components (notably ubiquinone).

We report here the results of an extensive kinetic study undertaken to characterize several aspects of the reactivity of both ubiquinol and cytochrome *c* with ubiquinol-cytochrome *c* reductase present in bovine heart submitochondrial particles (SMP); this may also contribute to a better understanding of the physiological implications of the enzymic reactions catalysed within biological membranes. The results strongly support the view that the reaction of ubiquinol is chemically, not diffusion-, controlled, whereas the reaction of cytochrome *c* has a strong diffusion-limited component.

A preliminary report on the effect of poly(ethylene glycol) on ubiquinol-1 oxidation by the *bc*₁ complex has been published (Fato et al., 1988a).

EXPERIMENTAL

Materials

Ubiquinones were gifts from Eisai Co., Tokyo, Japan. They were stored as solutions in absolute ethanol at -20°C at concentrations ranging between 10 and 40 mM, as determined spectrophotometrically at 275 nm using a molar absorption coefficient (oxidized minus reduced) of $12.5\text{ mM}^{-1}\cdot\text{cm}^{-1}$ (Degli Esposti et al., 1981). Ubiquinols were prepared according to

Rieske (1967) and stored in absolute ethanol under slightly acidic conditions as described by Degli Esposti et al. (1981); concentrations were determined at 290 nm with a molar absorption coefficient of $4\text{ mM}^{-1}\cdot\text{cm}^{-1}$. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Fluka, Buchs, Switzerland. Cytochrome *c* from horse heart (type III) was purchased from Sigma, St. Louis, MO, U.S.A. Soybean phospholipids (asolectin) were purchased from Associated Concentrates Inc. and purified according to Kagawa and Racker (1971) to remove neutral lipids. Poly(ethylene glycol) (PEG) (M_r 2000) was from Sigma. Cholesterol was from BDH, Poole, Dorset, U.K., and cholesteryl hemisuccinate was from Sigma.

Preparative procedures

SMP were prepared by sonic irradiation of bovine heart mitochondria in an MSE sonifier at 30 s intervals for a total time of 5 min, and kept frozen at -20°C . The particles were essentially broken membrane fragments and were permeable to cytochrome *c*, since the activity was not stimulated by detergents. In these particles, succinate evoked a slight quenching of 9-aminoacridine fluorescence (Casadio et al., 1974) that could be roughly calculated to correspond to $<5\text{--}8\%$ sealed inverted vesicles.

Ubiquinone-depleted mitochondrial membranes were prepared by pentane extraction of the lyophilized membranes according to the method of Szarkowska (1966), with some modifications (cf. Degli Esposti and Lenaz, 1982a). Ubiquinone-reconstituted mitochondria were prepared by transferring the pentane-extracted mitochondria together with the desired amounts of ubiquinones in pentane into a rotary evaporator in order to reincorporate ubiquinone into the particles (Norling et al., 1974).

Mitochondrial membranes containing different ubiquinone concentrations were also obtained by sonicating SMP together with phospholipid vesicles containing different amounts of ubiquinone-10 and subjecting them to a freeze-thaw cycle.

The *bc*₁ complex was isolated according to Degli Esposti and Lenaz (1982b) or by the method of Engel et al. (1980), and reconstituted in lipid vesicles by cholate dialysis (Degli Esposti et al., 1983). Cholesterol incorporation into mitochondrial membranes was performed using cholesterol-coated glass beads according to Coleman et al. (1978). Cholesteryl hemisuccinate was incorporated according to Shinitzky et al. (1979) by incubating the membranes with a medium containing 3.5% polyvinylpyrrolidone (M_r 40000; Sigma).

Analytical methods

Cytochrome *c*₁ concentration was determined from the ascorbate-reduced minus ferricyanide-oxidized spectrum in a Jasco 7850 spectrophotometer using a molar absorption coefficient of $17.5\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 552 nm minus 541 nm; cytochrome *b* concentration was determined from the dithionite-reduced minus oxidized spectrum using a molar absorption coefficient of $25\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 562 nm minus 576 nm. The content of *bc*₁ complex was routinely evaluated by dividing the cytochrome *b* content by two.

The ubiquinone content was determined by reverse-phase h.p.l.c. analysis (Tsai et al., 1985) after extraction with light petroleum (b.p. $40\text{--}60^{\circ}\text{C}$) and methanol using the method of Kröger and Klingenberg (1973). Separations were performed using a Spherisorb S5 ODS I $25\text{ cm}\times 0.46\text{ cm}$ column with a guard column containing the same material as the main column; the mobile phase was prepared by dissolving 7.0 g of $\text{NaClO}_4\cdot\text{H}_2\text{O}$ in 1000 ml of ethanol/water/70% HClO_4

(969:30:1, by vol.). The flow rate was 1 ml/min. Measurements were performed at 25 °C. The h.p.l.c. system was a Waters Data Module M730-Model 721 Programmable System Controller equipped with Lambda-Max Model 481 LC spectrophotometer.

Protein was determined by the biuret method of Gornall et al. (1949) and lipid phosphorus was determined according to Marinetti (1962); phospholipid content was calculated by multiplying lipid phosphorus by 25. Cholesterol was assayed according to Webster (1962).

Enzymic activity

Ubiquinol–cytochrome *c* reductase was determined essentially as described by Degli Esposti and Lenaz (1982b). To the assay cuvette was added 2–5 µg of submitochondrial particles per ml, corresponding to approx. 0.4–1 nM *bc*₁ complex, and cytochrome *c* at variable concentrations; in most experiments, the cuvette contained 50 mM KCl, 10 mM Tris/HCl buffer, pH 7.4, 1 mM EDTA and 2 mM KCN. This buffer was not at the pH optimum of the reductase, which is around 8, but offers a reasonable compromise for lowering the extent of chemical reduction of cytochrome *c* by the quinols and also, in terms of ionic strength, for preventing unspecific binding of cytochrome *c* to the enzyme (Speck and Margoliash, 1984). EDTA was included to remove the interference by cations of the electrostatic binding of cytochrome *c* to the enzyme, and KCN was added to inhibit cytochrome oxidase. These assay conditions have been considered as optimal throughout this work, since they avoid artifactual alterations of the typical kinetic behaviour of both cytochrome *c* and ubiquinols.

Use of a phosphate buffer (25 mM potassium phosphate, pH 7.5) allows somewhat higher reaction rates to be reached, but tends to enhance the chemical reaction, particularly when high cytochrome *c* concentrations are required, as in this study with membranous preparations (see Results).

The reaction was usually started with the quinol substrate, and cytochrome *c* reduction was monitored at 550 minus 540 nm in a Sigma–Biochem ZWS dual-wavelength spectrophotometer equipped with a rapid mixing apparatus (Degli Esposti and Lenaz, 1982b); a differential molar absorption coefficient of 19 mM⁻¹·cm⁻¹ was employed.

The chemical non-enzymic reaction was measured for each individual assay separately, either by mixing the substrates in the same medium in the absence of the enzyme, or by using the same enzyme concentration in the presence of an excess of both antimycin and myxothiazol, with similar rates. The evaluation of the chemical reaction and enzymic reaction in the same cuvette was avoided because it produces a mixture of reduced and oxidized cytochrome *c* that may affect the reactivity of this substrate.

When the isolated *bc*₁ complex was employed, the enzyme was used at 2–5 nM in the assay cuvette, which contained variable amounts of cytochrome *c* in 25 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA and 1 mM KCN.

The kinetic analysis was performed by the replot method of Cleland (1970) to obtain the absolute values of $V_{\max}$ and the K_m for the two substrates (ubiquinol and cytochrome *c*). When the absolute values of $V_{\max}/K_m$ were not necessary, determination of the kinetic constants was accomplished by varying ubiquinol at a fixed cytochrome *c* concentration (usually at a quasi-saturating concentration of 40–50 µM) and cytochrome *c* at a fixed concentration of ubiquinol (at quasi-saturating concentrations of 34 µM ubiquinol-1 and 16 µM ubiquinol-2). Thus the kinetic constants ($V_{\max}$ and K_m) throughout this report will carry the subscript either (Q) or (c) to indicate that they were obtained by

using ubiquinol or cytochrome *c* respectively as the variable substrate, under quasi-saturating concentrations of the other component. The catalytic constant $k_{\text{cat}} = V_{\max}/[bc_1 \text{ complex}]$.

Since ubiquinol interacts with the enzyme active site from within the lipid bilayer (Fato et al., 1988b), the concentration of ubiquinol in the lipid bilayer was expressed on a molar basis using the method of Fato et al. (1988b) for calculation of the kinetic constants ($K_{m(Q)}$ and $k_{\text{min.}(Q)}$). The method employs simple steady-state kinetic measurements at various membrane phospholipid fractional volumes in the assay medium; the replots of the apparent K_m , expressed as concentration in the total assay medium as a function of the membrane fractional volumes, yield both the true K_m in the membrane phase and the partition coefficients of ubiquinol.

Alternatively, the concentrations of ubiquinol corresponding to the true K_m were calculated using the partition coefficients established previously (Fato et al., 1986, 1988b; Battino et al., 1986). Generally, a partition coefficient of 2×10^4 (molar basis) was used for ubiquinol-2. In the calculations, ubiquinol was assumed to uniformly occupy the entire volume of the membrane phospholipids, taken to have an average density of 1 mg/µl, the volume of the protein was considered to be impermeable to ubiquinone, and no correction was applied for any bound form of the quinone. Since entrance of the quinol into the hydrophobic active site requires passing the phospholipid ‘annulus’ (Lenaz and Parenti Castelli, 1985), no distinction was made between free bilayer and boundary lipids as a solvent for ubiquinol substrate.

Biophysical determinations

Viscosity was determined as the product of the kinematic viscosity (η/ρ) measured with a KPG-Ubbelohde viscometer, and density (ρ).

Static fluorescence polarization of the probe DPH was measured in a Perkin–Elmer MPF4 spectrofluorometer equipped with polarization accessories. Although the steady-state polarization cannot discriminate between static and dynamic components [i.e. order parameters and viscosity respectively (Meier et al., 1982)], it was taken as a semi-quantitative indication of membrane fluidity (Shinitzky and Barenholz, 1978).

Determination of diffusion coefficients of ubiquinones by fluorescence quenching of 12-(9-anthroxyl)stearate (12-AS) was accomplished as described previously (Fato et al., 1986).

RESULTS

Kinetics of ubiquinol–cytochrome *c* reductase in SMP

Both ubiquinol-1 and ubiquinol-2 are convenient electron donors for investigating ubiquinol–cytochrome *c* reductase; the high catalytic-centre activity with ubiquinol-2 makes cytochrome *c* reduction rate-limiting and assures the best conditions for investigating a possible diffusion-limited step. On the other hand, using ubiquinol-1 as substrate makes ubiquinol oxidation rate-limiting.

Kinetic constants

We have determined the kinetic constants of ubiquinol–cytochrome *c* reductase in bovine heart SMP at 25 °C. Table 1 reports typical values obtained with SMP preparations using a KCl/Tris buffer; different preparations exhibited some variability, depending in part on the length of time for which the particles had been kept frozen. The use of a phosphate buffer (see the Experimental section) elicited somewhat higher rates, but the former buffer was preferred throughout the course of this study because the rate of non-enzymic chemical reaction was lower.

Table 1 Kinetic parameters of ubiquinol–cytochrome *c* reductase in bovine heart SMP at 25 °C

Activity was assayed in phosphate buffer (see the Experimental section). The k_{cat} values were obtained by dividing the V_{max} by the concentration of the bc_1 complex, calculated as one-half the concentration of cytochrome *b* (which is 0.44 nmol/mg of protein in the SMP used in this study). The values were obtained from the secondary plots of double-reciprocal plots (Figure 1, inset). Data with ubiquinol-1 are from Degli Esposti and Lenaz (1982b) and calculations therefrom.

Parameter		With ubiquinol-1	With ubiquinol-2
k_{cat}	(s^{-1})	2.2×10^2	3.7×10^2 *
$K_{m(Q)}$	(M, in water)	1.3×10^{-5}	1.4×10^{-6}
	(M, in lipids)	1.1×10^{-2}	2.7×10^{-2}
$K_{m(c)}$	(M)	1.4×10^{-5}	4.7×10^{-6}
$k_{min.(Q)}$	($M^{-1} \cdot s^{-1}$, in water)	1.7×10^7	2.6×10^8
	($M^{-1} \cdot s^{-1}$, in lipids)	1.0×10^4	1.3×10^4
$k_{min.(c)}$	($M^{-1} \cdot s^{-1}$)	1.5×10^7	7.7×10^7

* Values in the range 350–800 s^{-1} were typical.

Figure 1 Titrations of ubiquinol-2–cytochrome *c* reductase in bovine heart SMP

The variable substrate was ubiquinol-2 (Q_2H_2) at 11 fixed concentrations of cytochrome *c* from 2.92 to 78.3 μ M. For the sake of clarity, only the following concentrations are shown: ●, 2.92; ▲, 4.38; ○, 16.0; □, 78.3 μ M. The inset shows the secondary plots of the intercepts, from which the absolute V_{max} values can be obtained. The experiment was performed with KCl buffer (see the Experimental section). Note the absence of inhibition by oxidized cytochrome *c* even at the highest concentration used.

The values in Table 1 were obtained from secondary plots (Cleland, 1970) and are therefore authentic kinetic parameters for the bisubstrate reaction. As shown previously for the isolated complex (Degli Esposti and Lenaz, 1991), the parallel straight lines of the primary double-reciprocal plots of ubiquinol oxidation at different fixed cytochrome *c* concentrations (Figure 1) conform to a Ping Pong mechanism. The catalytic-centre activities found with the particulate enzyme are comparable to those of the isolated bc_1 complex, where values of 220 s^{-1} and 770 s^{-1} were found for ubiquinol-1 and ubiquinol-2 respectively.

A phenomenon observed previously with the isolated cytochrome *c* reductase is a striking inhibition by ferricytochrome *c* (Speck and Margoliash, 1984; Degli Esposti and Lenaz, 1991). This phenomenon is revealed by an increase in the slope of double-reciprocal plots obtained with variable ubiquinol when the concentration of cytochrome c^{+3} is raised above 20 μ M. This phenomenon was not observed in SMP (cf. Figure 1). Double-reciprocal plots were rigorously parallel even at concentrations of cytochrome *c* exceeding 50 μ M.

The SMP used in this study were routinely checked for leakiness by comparing enzyme activity in the presence and absence of detergents. Instead of the increase in activity expected if sealed particles were present, the detergent typically induced a small (0–20 %) decrease in activity. On the few occasions when latency was observed, the SMP were made 100 % leaky by repeated cycles of freezing and thawing to avoid the use of detergents.

Since in most cases substrate inhibition was not observed, the apparent V_{max} values obtained by ubiquinol titrations at fixed high cytochrome *c* concentrations ($> 40 \mu$ M) closely approach the real V_{max} values calculated from the secondary replot of the data, with values ranging from 350 to 800 s^{-1} .

Association rate constants ($k_{min.}$)

The values of $k_{min.(Q)}$ and $k_{min.(c)}$ obtained from the respective ratios of k_{cat}/K_m were quite high, and approached values suggestive of a diffusion-limited regime; nevertheless, when recalculated using membranous concentrations of ubiquinols (Fato et al., 1988b), the $k_{min.(Q)}$ values are considerably lower (Table 1).

No pre-steady-state kinetic constants are yet available for SMP; nevertheless, the values for the isolated beef heart bc_1 complex (Degli Esposti and Lenaz, 1991) allow us to directly compare $k_{min.}$ values with the respective bimolecular constants of an enzyme–substrate reaction. These are found to agree within the same order of magnitude. In the study with the isolated complex, $k_{min.(Q)}$ was found to be 30 % of k_1 for ubiquinol-2 and 82 % of k_1 for ubiquinol-1, whereas $k_{min.(c)}$ was 75 % of k_5 (Scheme 1). Presumably, similar relationships hold between the corresponding rate constants in the SMP also.

Scheme 1

Table 2 Comparison of the K_m values for ubiquinols and for cytochrome *c* in bovine heart SMP and in soluble bc_1 complex at 25 °C

Absolute values were obtained from secondary plots (Cleland, 1970; cf. also Degli Esposti and Lenaz, 1982b).

Substrate	K_m (μ M)	
	SMP	Soluble bc_1
Ubiquinol-1	13.4	24.2
Ubiquinol-2	1.4	6.7
Cytochrome <i>c</i>	4.7*	2.7

* In a previous study, using ubiquinol-1, the K_m for cytochrome *c* in cytochrome *c*-depleted mitochondria was 14.5 μ M (Degli Esposti and Lenaz, 1982b).

Table 3 Effect of endogenous ubiquinone-10 content on the kinetic parameters of ubiquinol–cytochrome *c* reductase

In Experiment 1, mitochondria were lyophilized and extracted with pentane (see the Experimental section). The control is represented by lyophilized mitochondria and the experimental samples are lyophilized mitochondria extracted to different extents. In Experiment 2, SMP were sonicated with phospholipids (in equimolar amounts with the endogenous phospholipid content) containing different levels of ubiquinone-10, obtained by co-sonicating phospholipids and ubiquinone-10. The control is untreated SMP. Note that in experiment 1, activity is expressed as the V_{max} (μ mol $\cdot$ min⁻¹ $\cdot$ mg⁻¹), while in Experiment 2 it is expressed as the k_{cat} (s⁻¹).

Experiment	Sample	Ubiquinone-10 content (nmol $\cdot$ mg ⁻¹)	Activity	K_m (μ M)	$k_{min(Q)}$ (μ M ⁻¹ $\cdot$ s ⁻¹)
1	Control	1.3	2.51	1.3	—
	Extracted	0.5	2.65	2.8	—
	Extracted	0.2	3.84	1.5	—
	Extracted	0.02	2.44	1.3	—
	Reconstituted	4.0	3.16	3.2	—
2	Control	4.5	620	1.8	340
	Treated	5.5	552	1.6	340
	Treated	10.1	736	1.6	450
	Treated	13.7	953	2.7	350

The K_m of 4.7 μ M found for cytochrome *c* in the membranous enzyme was slightly higher than the value of 2.7 μ M found in the isolated complex (cf. Degli Esposti and Lenaz, 1982b), whereas the K_m values for ubiquinol-1 and ubiquinol-2 were found to be lower in the membranous enzyme than in the soluble complex (Table 2).

Absence of product inhibition

In the Ping Pong mechanism for this enzyme (Speck and Margoliash, 1984; Kauten et al., 1987; Degli Esposti and Lenaz, 1991), the reaction scheme can be clearly divided into two parts, with the k_{min} for one substrate being completely independent of the kinetic rate constants of the other substrate (cf. Scheme 1). For a two-substrate enzyme, the kinetic analysis is simplified if no reversible steps occur under initial velocity conditions; this was verified by the negligible extent of product inhibition observed under the conditions of the assay. In fact, the presence of ubiquinone-1 at a concentration equal to that of cytochrome *c* had no effect on the oxidation of ubiquinol-1; this is the maximum amount of quinone that could accumulate in our steady-state assays. It should be noted, however, that excess ubiquinone causes product inhibition (Degli Esposti and Lenaz, 1991).

Endogenous ubiquinone

SMP contain endogenous ubiquinone-10 at a concentration of 2–5 nmol per mg of particle protein, corresponding to 4–10 mM in the lipid phase of the membrane (Lenaz, 1988). Under our assay conditions this endogenous quinone is presumably mostly in the oxidized state, and it might be questioned whether it exerts product inhibition.

The ubiquinone content was varied either by pentane extraction of lyophilized mitochondria or by sonicating SMP together with phospholipids containing different amounts of ubiquinone-10. Table 3 shows that the pentane-treated mitochondria had somewhat lower V_{max} values than untreated mitochondrial particles, probably as a result of solvent denaturation; on the other hand, the phospholipid-treated SMP had high activity. Nonetheless, in no case did a change in the content of endogenous ubiquinone-10 significantly change the enzyme kinetics.

Temperature-dependence

The temperature-dependence of the kinetic parameters of membrane-bound ubiquinol–cytochrome *c* reductase was investigated by varying each of the two substrates under quasi-saturating concentrations of the second substrate. Arrhenius plots of V_{max} extrapolated from varying ubiquinol-2 concentrations at a constant cytochrome *c* concentration of 50–100 μ M, and from varying cytochrome *c* concentrations at a constant ubiquinol-2 concentration of 16 μ M, were linear. This contrasts with previous findings of non-linear Arrhenius plots with a break centred at 18–20 °C when the cytochrome *c* concentration was kept at around 15 μ M (Lenaz, 1987).

The high concentrations of cytochrome *c* used in the experiments with varying ubiquinol-2 were required because the K_m for cytochrome *c* is strongly temperature-dependent (as found from the cytochrome *c* titrations at constant ubiquinol concentrations) and ranges from 5 to 20 μ M, contrary to the much lower values reported for the isolated bc_1 complex (Speck and Margoliash, 1984; Degli Esposti and Lenaz, 1982b, 1991). Consequently, performing ubiquinol titrations at the quasi-saturating cytochrome *c* concentrations that were usually employed with soluble bc_1 (< 15 μ M) progressively lowered the measured V_{max} , as the K_m for cytochrome *c* increased. The k_{cat} and K_m values obtained at each temperature were used to calculate the respective k_{min} value and to observe the temperature-dependence.

Activation energies

The temperature-dependence of $k_{min(Q)}$ and $k_{min(c)}$ is shown in Figure 2: the derived activation energy was significantly higher for $k_{min(Q)}$ [E_a = 24.7 kJ (5.9 kcal) $\cdot$ mol⁻¹] than for $k_{min(c)}$ [E_a = 5.9 kJ (1.4 kcal) $\cdot$ mol⁻¹].

The higher activation energy of $k_{min(Q)}$ compared with that of $k_{min(c)}$ must reflect additional kinetic events that are required prior to binding to the enzyme active site, including partition from the aqueous medium and diffusion into the lipid phase. It was therefore thought important to determine the temperature-dependence of $k_{min(Q)}$ based on the ubiquinol concentration in the lipid phase. The results of such an experiment are shown for ubiquinol-2 (Figure 3a); the corrected $k_{min(Q)}$ exhibited an E_a of 58.6 kJ (14 kcal) $\cdot$ mol⁻¹.

This steep temperature-dependence of $k_{min(Q)}$ resulted from the low-temperature coefficient of the true K_m for ubiquinol; the same experiment also yielded the partition coefficients of ubiquinol-2 at the various temperatures, revealing a decreased

Figure 2 Arrhenius plots of $k_{\min(Q)}$ and $k_{\min(c)}$ in bovine heart SMP

$k_{\min(Q)}$ for ubiquinol-2 (a) was obtained from ubiquinol-2 titrations at 50 μM cytochrome *c*; $k_{\min(c)}$ (b) was obtained from cytochrome *c* titrations at 15 μM ubiquinol-2. Activation energies: (a) 24.7 kJ (5.9 kcal) $\cdot\text{mol}^{-1}$, (b) 5.86 kJ (1.4 kcal) $\cdot\text{mol}^{-1}$.

Figure 3 Temperature-dependence of $k_{\min(Q)}$ and of ubiquinone diffusion in the membrane phase of SMP

The Arrhenius plot of $k_{\min(Q)}$ for ubiquinol-2–cytochrome *c* reductase in SMP was calculated for the reaction occurring in the membrane phase. The experiment was performed in phosphate buffer by means of ubiquinol-2 titrations at a fixed concentration of 40 μM cytochrome *c* at four different membrane fractional volumes [(1, 2, 4 and 8) $\times 10^{-3}$] at different temperatures. The true $k_{\min(Q)}$ values were calculated according to Fato et al. (1988b). The activation energy is 58.6 kJ (14 kcal) $\cdot\text{mol}^{-1}$. The inset shows the temperature-dependence of ubiquinone-3 diffusion measured by the fluorescence quenching of 12-AS as described by Fato et al. (1986). The data are expressed as bimolecular quenching constants in the membrane phase. Activation energy = 6.3 kJ (1.5 kcal) $\cdot\text{mol}^{-1}$.

partition of ubiquinol-2 into the membrane as the temperature is increased.

The activation energy for aqueous diffusion is very low and close to that found for the second-order rate constant for the association of cytochrome *c* with the enzyme. On the other hand, ubiquinol interacts with the enzyme by first partitioning from the aqueous phase into the membrane (Fato et al., 1988b). Conse-

quently, the activation energy for ubiquinol diffusion in the membrane was investigated by fluorescence quenching of the probe 12-AS by ubiquinones, as described previously (Fato et al., 1986). The temperature-dependence of ubiquinone diffusion in the membrane was extremely low [$E_a = 6.3$ kJ (1.5 kcal) $\cdot\text{mol}^{-1}$] (Figure 3b), and comparable to that for the aqueous diffusion of small molecules. It was significantly lower than that of $k_{\min(Q)}$, calculated either in the total assay medium or in the lipid phase. The rate-limiting step does not appear to be a diffusional step for ubiquinol in either the aqueous or lipid phases.

Figure 4 Ubiquinol–cytochrome *c* reductase titrations with ubiquinol as the variable substrate at a fixed low concentration of cytochrome *c* (3 μM)

The Eadie–Hofstee plot of ubiquinol-1–cytochrome *c* reductase in the isolated bc_1 complex (10 nM) in the solubilized form is shown in (a), whereas that in the complex reconstituted in asolectin vesicles is shown in (b). $\circ$, No addition; $\bullet$, +4.5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 1.5 $\mu\text{g}/\text{ml}$ valinomycin. In (c) is shown the titration of ubiquinol-2–cytochrome *c* reductase in the isolated bc_1 complex (4 nM); $\blacklozenge$, solubilized form; $\square$, reconstituted in asolectin vesicles without additions; $\blacksquare$, reconstituted as above, with the addition of 4.5 μM CCCP and 1.5 $\mu\text{g}/\text{ml}$ valinomycin.

Biphasic saturation kinetics

It has been reported that the saturation kinetics of enzymes associated with solid supports are often biphasic. This anomaly has been related to diffusional limitations (Goldstein, 1976), and we found previously that steady-state plots obtained from varying the ubiquinol-1 concentration at low cytochrome *c* concentrations are usually biphasic with membranous ubiquinol–cytochrome *c* reductase, but not with the soluble bc_1 complex (Degli Esposti and Lenaz, 1982a,b). On the other hand, plots obtained from varying the cytochrome *c* concentration as the primary substrate are usually linear.

We have now investigated the kinetics of ubiquinol–cytochrome *c* reductase in SMP with ubiquinol-2 as the donor substrate, and found similar biphasic plots at low cytochrome *c* concentrations (Figure 4). The plots extrapolate to a lower K_m at low ubiquinol concentrations and to a K_m of about one order of magnitude greater at high ubiquinol concentrations. As with ubiquinol-1 (Degli Esposti and Lenaz, 1982a,b), the soluble bc_1 complex gave linear double-reciprocal plots, whereas membranous bc_1 complex reconstituted in lipid vesicles also exhibited biphasic saturation kinetics (results not shown). The same behaviour was also found with ubiquinol-3 (results not shown). Decreasing the intrinsic rate of electron transfer through the complex by the use of inhibitors, by pre-reducing the complex with ascorbate or by using poor substrates such as duroquinol, restored the linear behaviour of the membrane reductase (results not shown).

Effect of medium viscosity

Noyes plots

The extent of diffusion control was investigated by changing the viscosity of the medium and using the Noyes equation:

$$1/k_{\text{obs.}} = 1/k_{\text{diff.}} + 1/k_c$$

where $k_{\text{obs.}}$ is the observed (experimental) $k_{\text{min.}}$, $k_{\text{diff.}}$ is the diffusion-limited component treated as a first-order process, and k_c is the chemically controlled component of the association rate constant (Noyes, 1961).

The viscosity of the aqueous medium was varied by using either PEG or sucrose, and ubiquinol–cytochrome *c* reductase kinetics were investigated by varying either ubiquinol or cytochrome *c*. PEG modifies the macroviscosity of the sample as measured, for instance, by conventional viscosity measurements. When diffusion is over large distances (e.g. micrometers), PEG can affect the measured diffusion constant by obstructing the path of the diffusant. Sucrose affects the micro- or true viscosity, and will affect short-range (nanometer) collisional processes, such as are measured by fluorescence quenching measurements.

Figure 5 Noyes plots of $k_{\text{min.}}^{-1}$ versus viscosity (obtained by varying the PEG concentration) of ubiquinol-2–cytochrome *c* reductase in SMP

●, Plot of $k_{\text{min.}(Q)}^{-1}$; ○, plot of $k_{\text{min.}(c)}^{-1}$.

Table 4 Effect of sucrose on the kinetic parameters of ubiquinol-2–cytochrome *c* reductase in bovine heart SMP

The 'true' K_m in the lipid phase and the partition coefficient P were calculated by titrating ubiquinol-2–cytochrome *c* reductase in SMP with ubiquinol-2 as the variable substrate and a fixed cytochrome *c* concentration of 40 μM , in the presence of different amounts of asolectin [corresponding to membrane fractions of $(1-8) \times 10^{-3}$], according to Fato et al., 1988b.

Parameter	Control	Sucrose (19%)
$k_{\text{cat.}}$ (s^{-1})	322	255
$K_{m(Q)}$ (M , in lipid phase)	1.1×10^{-2}	0.9×10^{-2}
$k_{\text{min.}(Q)}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$, lipids)	2.9×10^4	2.8×10^4
P (mol fraction)	4.1×10^4	1.8×10^4

Diffusional effects should always be demonstrable with sucrose, but sensitivity to PEG will depend upon the distance characteristics of the process studied.

A clear dependence of $k_{\text{min.}(c)}$ upon viscosity could be demonstrated with both viscosogens. The results with PEG are shown in Figure 5. Surprisingly, sucrose also appeared to produce a marked effect on $k_{\text{min.}(Q)}$, despite the small value of this parameter. This effect, however, appears to reflect changes in the value of the partition coefficient of ubiquinol between the aqueous and lipid phases. When $k_{\text{min.}(Q)}$ is calculated using the concentration of quinone in the lipid phase, the effect of sucrose concentration of $k_{\text{min.}(Q)}$ disappears, as expected (Table 4). No effect of PEG concentration on $k_{\text{min.}(Q)}$ was found. As PEG does not change the partition coefficient, the artifactual decrease in $k_{\text{min.}}$ found with sucrose is not produced by this viscosogen.

Extent of diffusion control

We have evaluated the extent of diffusion control from the Noyes equation (Table 5). Using PEG as a viscosogen and with ubiquinol-2, the chemically controlled association rate constant for cytochrome *c* was $6.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, almost equal to the diffusion-limited association rate constant at 1 $\text{mPa} \cdot \text{s}$. Thus the diffusion-limited component of $k_{\text{min.}(c)}$ was 52%; it was almost 100% with sucrose. On the other hand, for ubiquinol-2 the viscosity-independent component was $1.2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, while the diffusion-controlled constant at 1 $\text{mPa} \cdot \text{s}$ was about 7-fold greater, so that the extent of diffusion control was approx. 12%. It was higher (62%) with sucrose, but the experiment in Table 5 clearly shows that this effect reflects a change in partition coefficient.

Collisional efficiency

The collisional frequency, $k_{\text{coll.}}$, for a bimolecular diffusion-limited reaction [i.e. one in which the chemical reaction occurs with a rate constant $k_a \gg k_{-1}$ (dissociation of reactants)] is given by the Smoluchowski relation (Smoluchowski, 1917):

$$k_{\text{coll.}} = 4\pi RDN/1000$$

where R is the encounter distance of the two molecules, taken as the sum of the radii of the two interacting molecules or sites, D is the sum of the diffusion coefficients in $\text{cm}^2 \cdot \text{s}^{-1}$ and N is Avogadro's number. The collisional efficiency was calculated from the ratio of the diffusion-limited association rate constant to the collisional frequency calculated from the Smoluchowski relation.

The encounter radius of cytochrome *c* and the enzyme in the membrane was taken as twice the radius of cytochrome *c*, 33.2 Å (where 1 Å = 10^{-10} m) (Wherland and Gray, 1977), and the

Table 5 Effect of viscosogens on the kinetic parameters of ubiquinol–cytochrome *c* reductase in SMP and on the extent of diffusion control

Parameters are obtained from Noyes plots, as in Figure 4. The second-order rate constants are expressed as molarity in the total assay medium, being derived from experiments where the viscosity of the water phase was varied. The data with ubiquinol-1 as the donor substrate and using PEG as viscosogen are taken from Fato et al. (1988b).

Parameter	With ubiquinol-1		With ubiquinol-2	
	Ubiquinol-1	Cytochrome <i>c</i>	Ubiquinol-2	Cytochrome <i>c</i>
Using PEG				
$k_{\min}$ ($M^{-1} \cdot s^{-1}$)	6.8×10^6	3.2×10^7	1.1×10^8	3.3×10^7
k_c ($M^{-1} \cdot s^{-1}$)	8.0×10^6	2.0×10^8	1.2×10^8	6.9×10^7
k_{diff} ($M^{-1} \cdot s^{-1}$)	6.7×10^7	3.8×10^7	8.8×10^8	6.4×10^7
Diffusion-limited component (%)	11	84	12	52
Using sucrose				
$k_{\min}$ ($M^{-1} \cdot s^{-1}$)	3.0×10^6	2.3×10^7	1.1×10^8	3.6×10^7
k_c ($M^{-1} \cdot s^{-1}$)	4.6×10^6	4.8×10^7	2.9×10^8	1.1×10^9
k_{diff} ($M^{-1} \cdot s^{-1}$)	8.8×10^6	4.5×10^7	1.8×10^8	3.7×10^7
Diffusion-limited component (%)	34	51	62	97

Table 6 Effect of cholesterol incorporation on the kinetics of ubiquinol–cytochrome *c* reductase in bovine heart SMP and in isolated *bc₁* complex reconstituted with asolectin

In Expt. 1, cholesterol was incorporated into SMP by the method of Coleman et al. (1978). The kinetic values were obtained from Lineweaver–Burk plots of ubiquinol-2–cytochrome *c* reductase activity using ubiquinol-2 as the variable substrate at a fixed concentration of cytochrome *c* ($40 \mu M$), and are the averages of three separate experiments. The 'true' K_m in the lipid phase were calculated using a partition coefficient (P) for ubiquinol-2 of 2×10^4 (molar units). The assay cuvette contained $2.4\text{--}3 \mu g$ of SMP protein/ml, corresponding to $1.2\text{--}1.5 \mu g$ of phospholipids. The volume of the membrane (lipid) phase was assumed to be $1 \mu l/mg$ (including cholesterol, when present). The ubiquinol-2 concentration in the lipid phase (Q_m) was calculated from Fato et al. (1988b):

$$Q_m = (P \times Q_{\text{total}}) / [(P \times \alpha) + (1 - \alpha)]$$

where α is the relative volume of the lipid phase with respect to the total assay medium and P is the partition coefficient of ubiquinol-2 in molar units. k_{coll} values are bimolecular fluorescence quenching constants of the probe 12-AS by ubiquinone-3 in the SMP measured as described by Fato et al. (1986). The values reported are the true constants in the membrane lipids. Expt. 2 was performed on a *bc₁* complex purified by the method of Engel et al. (1980). The assay cuvette contained $6.6 \text{ nM } bc_1$ complex reconstituted at a phospholipid/protein ratio of 15:1 (w/w), with or without cholesterol at a molar ratio of 1:2 with the phospholipids. The volume of the lipid phase was calculated to be $0.025 \mu l/ml$ in the case of asolectin and $0.032 \mu l/ml$ in the case of asolectin plus cholesterol. The ubiquinol-2 concentration in the lipid phase, Q_m , was calculated as for Expt. 1 in SMP, using the same partition coefficient for ubiquinol-2.

Expt. 1 (SMP) Parameter		Cholesterol	
		1st level	2nd level
Cholesterol content (nmol/mg)	35.5	86.0	138.0
$10^{-9} \times k_{\text{coll}}(\text{app.})$ ($M^{-1} \cdot s^{-1}$)	2.0	1.6	1.4
k_{cat} (s^{-1})	240	254	259
$K_{m(Q)}$ (mM, in lipid phase)	29.8	31.1	34.3
$10^{-4} \times k_{\min(Q)}$ ($M^{-1} \cdot s^{-1}$, in lipid phase)	0.80	0.82	0.76
Expt. 2 (reconstituted <i>bc₁</i>) Parameter		Cholesterol	
		Control	
k_{cat} (s^{-1})	100	140	
$K_{m(Q)}$ (M, in lipids)	3.95×10^{-2}	5.6×10^{-2}	
$k_{\min(Q)}$ ($M^{-1} \cdot s^{-1}$, in lipids)	2.5×10^3	2.5×10^3	
DPH fluorescence polarization	0.09	0.20	

three-dimensional diffusion coefficient of cytochrome *c* in water at high ionic strength was taken as $1.3 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$ at 20°C (Margoliash and Listgarten, 1962); the two-dimensional diffusion coefficient of the *bc₁* complex in the membrane was considered to be negligible (Gupte et al., 1984). From the Smoluchowski equation, a k_{coll} of $3.4 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cytochrome *c* and the *bc₁* complex in the membrane was calculated; the resulting collision efficiency from the diffusion-limited component, k_{diff} , of $k_{\min(Q)}$ was 1.9%.

A similar calculation for ubiquinol requires transformation of the rate constants using membrane concentrations of the quinol.

Using a partition coefficient for ubiquinol-2 of 2×10^4 M from water to membrane (Fato et al., 1988b), the k_{diff} for ubiquinol-2 in the membrane is $4.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. Assuming an encounter radius of $8 \text{ \AA}$ (Fato et al., 1986) and a diffusion coefficient of $4 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ for ubiquinones in the membrane (Lenaz, 1988), k_{coll} , calculated from the Smoluchowski relation, is $2.5 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. The collisional efficiency for the diffusion-limited component that results from the comparison of $k_{\min(Q)}$ and $k_{\text{coll}(Q)}$ is very low (approx. 0.02%). Even were $D_{(Q)}$ two orders of magnitude smaller, as proposed by Hackenbrock et al. (1986), the collisional efficiency would still be $< 2\%$.

Effect of membrane viscosity

Because ubiquinols must diffuse in the lipid hydrophobic medium prior to reaching the active site of ubiquinol cytochrome *c* reductase, we have investigated $k_{\min.(Q)}$ under conditions in which ubiquinol diffusion is expected to be impaired.

Incorporation of cholesterol

Cholesterol was introduced into SMP by the method of Coleman et al. (1978); this led to a small but significant decrease in the diffusion-limited bimolecular quenching constant of the probe 12-AS by ubiquinone-3 (Table 6, Expt. 1). In spite of this decrease of ubiquinone diffusion, there was no concomitant change of any significance in $k_{\min.(Q)}$.

In these experiments the incorporation of cholesterol into the membrane was relatively modest, and we have therefore investigated its effect under more drastic conditions in a reconstituted system.

Isolated bc_1 complex was incorporated into lipid vesicles containing cholesterol at a 2:1 molar ratio of phospholipid to cholesterol and the kinetic constants of the membranous enzyme in the presence and the absence of cholesterol were determined (Table 6, Expt. 2). In spite of the increase in membrane order, as implied by the increase in fluorescence polarization of the probe DPH, there was no significant change in $k_{\min.(Q)}$. Under similar conditions it was shown (Fato et al., 1986) that the diffusion coefficients of ubiquinones are greatly decreased by the sterol, as measured by fluorescence quenching of anthroylstearate probes. The effect of cholesterol incorporation on the temperature-dependence of $k_{\min.(Q)}$ is shown in the form of an Arrhenius plot (Figure 6). No systematic difference between the two sets of data was apparent, with an average activation energy of 29.3 kJ (7.0 kcal) $\cdot$ mol $^{-1}$.

A popular agent employed for decreasing membrane fluidity is cholesteryl hemisuccinate (Shinitzky et al., 1979). Progressive incorporation of this sterol derivative into SMP produced the expected increases in DPH fluorescence polarization. Unfortunately, the use of cholesteryl hemisuccinate as a viscosogen was found to yield unspecific inhibition, as shown, for example, by the strong inhibition of cytochrome *c* oxidase activity which does not interact with ubiquinone.

Figure 6 Temperature-dependence of $k_{\min.(Q)}$ of ubiquinol-2–cytochrome reductase in asolectin-reconstituted bc_1 complex in absence and in presence of cholesterol

●, No cholesterol; ○, cholesterol at a molar ratio of 2:1 with the phospholipids. The values were calculated from the $V_{\max}$ and the K_m obtained by double-reciprocal plots at different temperatures, using ubiquinol-2 as the variable substrate and cytochrome *c* (40 μ M) as the fixed substrate. The K_m values were not corrected for the partition of ubiquinol-2 in the membrane and are therefore expressed as μ M in the assay medium. Assay conditions are as in Table 4.

DISCUSSION

The parameter $k_{\text{cat.}}/K_m$, the minimum second-order rate constant for the reaction of an enzyme with a substrate, reflects the rate constants of all processes up to and including the first irreversible step of the reaction studied (Hardy and Kirsh, 1984). Although this parameter is normally exploited in reactions involving the conversion of a single substrate to products, Scheme 1 shows that it is equally valid for reactions involving two substrates, at least those conforming to the Ping Pong mechanism.

Most investigations on diffusion-limited steps in enzymic reactions have so far concerned soluble enzymes catalysing monosubstrate or pseudomonosubstrate reactions; application of the results obtained with those enzymes to bisubstrate enzymatic reactions is not necessarily straightforward.

Kinetics of ubiquinol–cytochrome *c* reductase in SMP

Isolated ubiquinol–cytochrome *c* reductase catalyses a two-substrate reaction that follows a two-site Ping Pong mechanism (Speck and Margoliash, 1984; Kauten et al., 1987; Degli Esposti and Lenaz, 1991). We have now shown that this mechanism also occurs in the membrane-bound form of the reductase, thus substantiating most of the observations obtained with the solubilized complex; these are summarized in Scheme 1. In principle, there are two sites at which ubiquinol can be oxidized, according to the Q-cycle mechanism (Berry and Trumpower, 1985); nevertheless, in the steady-state mechanism of Scheme 1, k_1 represents the rate-limiting step of quinol oxidation by the enzyme and can equally describe oxidation of ubiquinol at either centre i or o (Degli Esposti and Lenaz, 1991), although only the reaction at centre o can lead to cytochrome *c* reduction in the steady state.

The absolute catalytic-centre activity, extrapolated from secondary plots of the intercepts of initial double-reciprocal plots using ubiquinol-2 as the variable substrate, are comparable for SMP and the isolated complex. Occasionally, lower values are found, however; these can have a number of explanations, such as environmental differences (phospholipids versus detergents etc.) affecting the enzyme structure. For example, the difference redox potential between cytochrome *c* and cytochrome c_1 is positive in the isolated complex (Kim et al., 1987) but is negative in the membrane-bound form (Nicholls, 1976).

The $k_{\min.(Q)}$ and $k_{\min.(c)}$ values measured were within the range previously observed by us (Degli Esposti and Lenaz, 1991) in the isolated bc_1 complex; in particular, the $k_{\min.(c)}$ value of $7.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ found in SMP (this study) compares with a directly measured value for k_5 (see Scheme 1) of $3.8 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the isolated complex. Previously, Yu et al. (1973) obtained a rate of $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the reaction of ferricytochrome *c* with ferrocyanide c_1 , both with the isolated c_1 and with detergent-stabilized succinate–cytochrome *c* reductase. This low value appears to be a result of the high ionic strength employed by Yu et al. (1973).

A striking result is the lack of substrate inhibition exerted by cytochrome *c* (cf. Figure 1), in contrast with the inhibition observed in the soluble heart enzyme (but not in the yeast enzyme). An explanation for this inhibition is provided by the study of Degli Esposti and Lenaz (1991), in the reactivity of high concentrations of cytochrome *c* with the oxidized Rieske protein in the complex, which would ‘pull’ additional electrons from ubiquinol, increasing the apparent K_m for the donor substrate. This substrate inhibition by cytochrome *c* would therefore explain the high turnovers extrapolated from the quinol titrations that

are measured when the reductase is assayed at very high concentrations of cytochrome *c*. The lack of substrate inhibition in SMP can be interpreted as the result of greater integrity of the structure of the enzyme in the intact membrane in the absence of detergents.

SMP contain endogenous ubiquinone-10 at concentrations close to the K_m of those enzymes using ubiquinone or ubiquinol, including ubiquinol–cytochrome *c* reductase (Lenaz, 1988). This finding raises the question of a possible product inhibition by oxidized endogenous ubiquinone-10 on ubiquinol–cytochrome *c* reductase activity. Our data exclude this possibility, as neither the V_{max} , nor the K_m for ubiquinol-2 were significantly affected by the ubiquinone content of the membrane within the range 20 pmol–10 nmol/mg of mitochondrial protein (Table 3; these values translate to approx. 40 μ M–20 mM ubiquinone in the membrane lipid). This lack of inhibition is to be expected under our experimental conditions. In fact, if the K_i of 8.9 μ M found for oxidized ubiquinone-2 in the isolated bc_1 complex (Degli Esposti and Lenaz, 1991) is calculated as ubiquinone-2 concentration in the lipid phase, using the partition coefficient for this quinone (Fato et al., 1986) and the relevant equation (Table 6; Fato et al., 1988b), a value of > 100 mM is obtained. If the same K_i is applicable to ubiquinone-10, then the physiological concentration of 10 mM in the membrane would exert only a negligible inhibition, as indeed we have found experimentally.

Reassured by these considerations, we performed a series of experiments aimed at detecting possible diffusion-limited steps in the reaction catalysed by ubiquinol–cytochrome *c* reductase *in situ* in SMP from beef heart.

Differences between soluble and membrane-bound enzyme

The K_m for cytochrome *c* in both SMP and mitochondria is higher than that found in the solubilized complex (Degli Esposti and Lenaz, 1982b), whereas the K_m for ubiquinols is lower. The k_{cat}/K_m for cytochrome *c* is correspondingly lower in the membranous enzyme. This effect is not related to the sidedness of the membrane, but to the membrane-bound state *per se*, since it occurs both in mitochondria and in SMP. It is to be emphasized that SMP used in this study are permeable to cytochrome *c*, being open fragments of the mitochondrial membrane.

It is decidedly relevant that, for immobilized enzymes, diffusional limitations were reported to lead to an increase in K_m for the more limiting substrate, accompanied by a decrease of K_m for the co-substrate (Engasser and Hisland, 1978).

Temperature-dependence

The large temperature sensitivity of the K_m of cytochrome *c*, when combined with the large temperature-dependence of the V_{max} , results in a low temperature coefficient of $k_{min.(c)}$, which is similar to that found for the aqueous diffusion of solutes, i.e. 5.86 kJ (1.4 kcal)·mol⁻¹. On the other hand the activation energy of $k_{min.(Q)}$, calculated considering the ubiquinol-2 concentration either in the total assay medium or in the membrane phase [i.e. 24.7 kJ (5.9 kcal)·mol⁻¹ (Figure 2) and 58.6 (14 kcal)·mol⁻¹ (Figure 3) respectively], is higher than the values described for aqueous diffusion and also for lipidic diffusion of quinones, as determined by fluorescence collisional quenching [6.9 kJ (1.65 kcal)·mol⁻¹, cf. Figure 3 and Fato et al., 1986].

The high value for the activation energy of $k_{min.(Q)}$ is a strong indication that the association rate constant of ubiquinol-2 in the lipid phase is governed by chemical activation only. This conclusion agrees with that of Wang et al. (1987) regarding the

activation energy of cytochrome *b* reduction by ubiquinol-10 in *Rhodobacter sphaeroides* chromatophores.

The temperature studies revealed that the break in the Arrhenius plot of the V_{max} , previously found with this enzyme in the membrane-bound form (Lenaz, 1987) originates in the anomalously steep temperature coefficient of the K_m for cytochrome *c*. Ubiquinol titrations in the membrane-bound bc_1 complex of the enzymic activity in the presence of cytochrome *c* concentrations which are saturating for the soluble bc_1 complex clearly underestimate the apparent V_{max} in a manner that progressively increases with increasing temperature. The break in the Arrhenius plot of the apparent V_{max} is clearly due to this effect, since an increase in cytochrome *c* concentration abolishes the discontinuity in the Arrhenius plot (results not shown).

Saturation kinetics

The steady-state kinetics of membrane-bound ubiquinol–cytochrome *c* reductase result in biphasic Lineweaver–Burk (or Eadie–Hofstee) plots with ubiquinol titrations at low cytochrome *c* concentrations (Figure 4).

Deviations from linearity for such plots are common with insoluble enzymes (Havekes et al., 1974; Campbell and Hornby, 1975; Engasser et al., 1977; Lluís, 1984) and may reflect a diffusion-limited step (Goldstein, 1976). Although the explanation of the downward curvature of the double-reciprocal plots with ubiquinol is not straightforward in a diffusion-limited model, it may be empirically compatible with diffusional limitation by cytochrome *c*. Analogous patterns are exhibited by immobilized enzymes (Havekes et al., 1974; Campbell and Hornby, 1975; Lluís, 1984), although a clear demonstration that the behaviour was actually a manifestation of diffusion control was not given in these studies. Other interpretations for the biphasic saturation kinetics of ubiquinol–cytochrome *c* reductase have been given previously, such as the existence of two sites for ubiquinol (Degli Esposti and Lenaz, 1982a,b) and an interaction of ubiquinone with a dimeric form of the enzyme (Nalecz and Azzi, 1985).

It might be argued that non-linear plots reflect two modes of reaction of the ubiquinol, e.g. reaction from both the aqueous and lipid phases. However, the observation that such behaviour is only observed at low cytochrome *c* concentrations is clearly inconsistent with such a conclusion, as one would expect this non-linear behaviour to be maintained, or even enhanced, at higher concentrations of cytochrome *c*, when the ubiquinol half-reaction becomes the only rate-limiting process. Moreover, the same behaviour is obtained with ubiquinols of different chain lengths and hence hydrophobicities.

Effect of medium viscosity

When PEG is used as a viscogenic agent, the viscosity dependence of $k_{min.}$ is significant only for cytochrome *c*, being marginal for ubiquinols. On the other hand, when sucrose is used to increase the medium viscosity, there is a significant decrease of $k_{min.(Q)}$ when ubiquinol-2 is the reducing substrate. It has been reported that polymeric viscosogens do not interfere with short-range diffusion of small molecules (Phillips et al., 1977) and that PEG is excluded from liposome (and presumably membrane) surfaces (Arnold et al., 1990). It is therefore likely that diffusion of the bulky cytochrome *c* molecule is slowed by both PEG and sucrose, whereas the diffusion of ubiquinol in water is slowed only by the low- M_r viscosogens.

By performing ubiquinol plots at constant cytochrome *c* concentration in the presence of different phospholipid concen-

trations (Fato et al., 1988b), we could calculate both the K_m of ubiquinol expressed as the concentration in the lipid phase and the partition coefficient of ubiquinol. The K_m (and hence $k_{min.}$) are not affected by sucrose (Table 4) when expressed using the concentration in the lipid phase, but the partition coefficient into the membrane decreases. This could be the result of a lower diffusion coefficient of the quinol, or of changes at the lipid–water interface induced by sucrose, a well-known dehydrating agent (cf. Hackenbrock et al., 1986), or of direct quinol–sucrose interactions lowering the chemical activity of the quinol in the aqueous phase.

Even if aqueous diffusion of ubiquinol might result in a rate limitation for $k_{min.(Q)}$, this effect cannot be considered to be of physiological importance, because of the completely hydrophobic character of the natural ubiquinone.

From Noyes plots (Figure 5), the value of the diffusion-limited component can be determined and the collisional efficiency calculated as the fraction of collisions that result in a reaction (i.e. the ratio of the diffusion-controlled component of $k_{min.(c)}$ to the collisional frequency calculated from the diffusion coefficient). The efficiency thus determined is low, but comparable with that found for other enzymes (Hasinoff and Davey, 1987).

Gupte and Hackenbrock (1988a,b) calculated the collision frequencies and collision efficiencies of cytochrome *c* with bc_1 complex at the surface of the mitochondrial inner membrane at various ionic strengths in a lateral, pseudolateral and three-dimensional diffusion mode. The collision efficiencies in their study ranged between 1.8 and 6.1 %, in agreement with the value of 1.9 % in the present investigation.

Effect of membrane viscosity

Direct incorporation of cholesterol into SMP induces a small but significant decrease in the bimolecular quenching constant of the membrane probe AS by ubiquinone-3, indicating a decreased diffusion coefficient of this quinone; nevertheless, no significant change occurs in any of the kinetic parameters (K_m , $V_{max.}$ and $k_{min.}$). Since the viscosity change induced by cholesterol in SMP is rather small, and no success was achieved in attempting to introduce larger amounts of the sterol into the membrane, we have reconstituted the isolated bc_1 complex in liposomes containing large proportions of cholesterol; while the inclusion of cholesterol lowers the diffusion coefficient of ubiquinones, it fails to change the kinetic parameters in a significant way.

Other studies have demonstrated that a decrease in the fluidity of the mitochondrial membrane in *Saccharomyces cerevisiae* induced by a different temperature regime during growth decreases the lateral diffusion coefficient of ubiquinone, but does not affect the kinetics of ubiquinol–cytochrome *c* reductase (in particular, the $k_{min.(Q)}$ is unaffected) (M. Cavazzoni, J. Svoboda, A. De Santis and G. Lenaz, unpublished work).

Conclusions

The results reported in this paper demonstrate that the catalytic activity of ubiquinol–cytochrome *c* reductase in mitochondrial membranes is limited by the aqueous diffusion of cytochrome *c* but not by the membrane diffusion of short-chain ubiquinols.

Although the natural substrate of the enzyme is ubiquinol-10 in mammalian mitochondria, and the interaction of the substrate with the enzyme could not be tested here, there is no reason to believe that the reaction of ubiquinol-10 will be any different from that of the short-chain ubiquinones. In particular, it is known that the kinetic mechanism, the sensitivity to inhibitors, the variation in activity with temperature and the capacity to

support phosphorylation and proton uptake are essentially similar with short- and long-chain ubiquinol homologues, making it quite unlikely that there are different sites of reaction for ubiquinol-2 and ubiquinol-10 (cf. Fato et al., 1988b, and references therein). Moreover, the redox centres (especially the cytochrome *b* haems) are buried in the hydrophobic core of the protein (Saraste, 1984; Degli Esposti et al., 1989).

While some of our results, in particular the effects of viscosity and the biphasic saturation kinetics, may suggest that short-chain ubiquinols react from the aqueous phase, we have provided alternative explanations which favour reaction from the lipid phase.

Although the $k_{min.(Q10)}$ of the mitochondrial bc_1 complex is not known, in a bacterial bc_1 complex (from *R. sphaeroides* chromatophores) k_1 was found to be $3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Snozzi and Crofts, 1984). Furthermore, the $k_{cat.}/K_m$ ratio for ubiquinol-10 can be approximated from the K_m value of Zhu et al. (1982) for total ubiquinone in bovine heart SMP of 0.6 nmol/mg of protein, corresponding roughly to 1.2 mM in the lipid bilayer. The $k_{cat.}$ with ubiquinol-10 can be obtained by the maximal observed turnover of NADH–cytochrome *c* reductase and of NADH–ubiquinone reductase by applying the pool equation of Kröger and Klingenberg (1973). A value of 370 s^{-1} at 20°C could be obtained (Lenaz, 1988). From these figures, a value of $3.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ is calculated for $k_{min.(Q10)}$, in surprising agreement with the value of k_1 for ubiquinol-10 in bacterial chromatophores (Snozzi and Crofts, 1984).

It cannot be ruled out at this stage that the somewhat higher value of $k_{min.(Q)}$ with ubiquinol-10 than with ubiquinol-2 may impose some extent of diffusion control on the enzymic reaction. However, it is unlikely that such an effect, if present, could be significant. It is distinctly relevant that titrations of the extent of cytochrome *b* reduction with ubiquinol-10 (in the presence of antimycin) at different temperatures led Wang et al. (1987) to conclude that diffusion control was also not present in bacterial chromatophores.

These data represent a necessary basis for further investigations on the possible diffusion-limited steps of integrated electron transfer via the pool behaviour of ubiquinone and cytochrome *c*, where the average distance between complexes is a crucial factor in establishing the extent to which diffusion control may be present (Chazotte and Hackenbrock, 1988).

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