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A simple method for the determination of the kinetic constants of membrane enzymes utilizing hydrophobic substrates: ubiquinol cytochrome *c* reductase

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We have devised a method to determine the true K_m of membrane enzymes for hydrophobic substrates dissolved in lipid bilayers, and the lipid/water partition coefficients, by simple steady-state kinetic measurements at varying membrane phospholipid fractional volumes in the assay medium. The method has been applied to mitochondrial ubiquinol cytochrome *c* reductase, using short-chain ubiquinols as reductants at saturating cytochrome *c*. The partition coefficients of the quinols, as obtained by this method, are in good agreement with those determined directly by other procedures; K_m values obtained by this method, when expressed as concentrations in the lipid bilayer, are in the millimolar range. The kinetics of the ubiquinol analog duroquinol are independent of phospholipid concentration, as expected from its partition coefficient close to unity.

Introduction

Several membrane-bound enzymes utilize hydrophobic substrates which react with the active site from within the lipid bilayer. The kinetic properties of these enzymes are usually complicated by incomplete knowledge of the substrate concentration in the lipid bilayer. Even greater problems are found when partly water-soluble substrate analogs are used to overcome the difficulties in handling the water-insoluble natural substrates:these analogs partition from the aqueous medium into the lipid hydrophobic phase, and their concentration in the lipid bilayer is a function of their partition coefficients and of the relative fraction of the membrane volume in the total assay medium.

In most cases, the kinetic constants of these enzymes are calculated taking into account the total substrate concentration in the heterogeneous assay mixture, rather than the true substrate concentration in the membrane. This can lead to ambiguity in comparisons of the specificity of substrates having different partition coefficients and in establishing the presence of diffusion-limited components of the reactions.

Methods to determine the true kinetics of association reactions in lipid bilayers have been developed for fluorescence collisional quenching [1,2]; such methods allow the calculation of both the partition coefficients of the quencher and the true biomolecular quenching (rate) constants from simple fluorescence determinations at different membrane fractional volumes.

Abbreviation: PL, phospholipid.

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We have implemented a simple method for determining both the Michaelis constants of hydrophobic substrates of membrane-bound enzymes and the partition coefficients of the substrates into the membrane by performing saturation kinetics experiments at different membrane fractional volumes. The method has been applied to ubiquinol cytochrome *c* reductase (E.C. 1.10.2.2) [3], both in situ in submitochondrial particles and in reconstituted lipid vesicles, using exogenous short-chain ubiquinol homologs (ubiquinol-1 and ubiquinol-2) as the reducing substrates. The partition coefficients of the quinols, as obtained by this method, are in good agreement with those directly determined by other procedures; K_m values obtained by this method, when expressed as concentrations in the lipid bilayer, are in the millimolar range.

Materials and Methods

Bovine heart submitochondrial particles were prepared as described elsewhere [4]. A mitochondrial fraction containing the bc_1 complex (fraction R_4B) was prepared according to Hatefi [5]; the fraction contained 0.8 nmol cytochrome c_1/mg protein. Phospholipid vesicles were prepared [6] by sonication of purified soybean phospholipids (Asolectin, from Associated Concentrates, New York). Proteoliposomes containing bc_1 complex were obtained by cholate dialysis of the fraction R_4B with different proportions of phospholipids, as in Ref. 7. Ubiquinols were prepared from stock solutions of the corresponding ubiquinones (kindly donated by Eisai Co., Tokyo) by the method of Rieske [8] and the quinol concentration was determined at 275 nm in ethanol using the extinction coefficient of 12 $mM^{-1} \cdot cm^{-1}$, oxidized minus reduced [9]. Ubiquinol cytochrome *c* reductase activity was assayed as described previously [10], but using a Sigma-Biochem dual-wavelength spectrophotometer equipped with a rapid mixing device, and following the reduction of cytochrome *c* at 550 minus 540 nm. The cytochrome c_1 concentration was determined from the dithionite-reduced minus ferricyanide-oxidized spectrum in a Perkin-Elmer 559 spectrophotometer, using an extinction coefficient of 20 $mM^{-1} \cdot cm^{-1}$ at 553 minus 540 nm [11].

Theory

The measurement of the K_m of membrane enzymes for their substrates, S, in lipid bilayers requires knowledge of the substrate concentration in the lipid bilayer. At low substrate concentrations, the concentration of S in each phase is described by a lipid-water partition coefficient *P*:

$$P = \frac{[S]_L}{[S]_W} \tag{1}$$

where the subscripts L and W refer to the membrane lipid phase and the water phase, respectively.

The total (T) concentration of S added partitions between the water and membrane phases:

$$[S]_{added} V_T = [S]_L V_L + [S]_W V_W \tag{2}$$

By defining

$$\alpha = \frac{V_L}{V_T} \tag{3}$$

to be the volume fraction of the membrane, and substituting $[S]_W = [S]_L/P$, we obtain:

$$[S]_L = \frac{P[S]_{added}}{P\alpha + 1 - \alpha} \tag{4}$$

The Michaelis–Menten equation for an enzyme using a single substrate present in the membrane phase can now be rewritten as:

$$v = \frac{V_{max}[S]_L}{K'_m + [S]_L} \tag{5}$$

where the subscript L again refers to the membrane lipid phase, and K'_m is the true K_m of the enzyme for the substrate dissolved in the membrane. If $[S]_L$ is expressed in $mol \cdot l^{-1}$ of membrane lipids, K'_m will have the same dimensions.

The corresponding Lineweaver–Burk equation becomes:

$$\frac{1}{v} = \frac{1}{[S]_L} \frac{K'_m}{V_{max}} + \frac{1}{V_{max}} \tag{6}$$

Substitution of Eqn. 4 into Eqn. 6 gives:

$$\frac{1}{v} = \frac{P\alpha + 1 - \alpha}{P[S]_{added}} \frac{K'_m}{V_{max}} + \frac{1}{V_{max}} \tag{7}$$

Putting

$$\frac{(P\alpha + 1 - \alpha) K'_m}{P} = K_{app} \tag{8}$$

where K_{app} is the apparent K_m of the enzyme for the substrate in the total solution, we obtain:

$$\frac{1}{v} = \frac{1}{[S]_{added}} \frac{K_{app}}{V_{max}} + \frac{1}{V_{max}} \tag{9}$$

which is the traditional form of the double-reciprocal equation.

Rearranging Eqn. 8 gives a simple relation for K_{app}, viz:

$$K_{app} = \alpha\left(K'_m - \frac{K'_m}{P}\right) + \frac{K'_m}{P} \tag{10}$$

Thus a plot of K_{app} vs. α allows the determination of both the partition coefficient and the true K_m of the enzyme.

For the determination of α it is assumed that only the phospholipids (and not the proteins) are available for dissolving the substrate: thus a membrane fraction equivalent to 1 mg/ml phospholipids (having a density close to 1 g/ml) corresponds to $\alpha = 10^{-3}$, irrespective of the protein content.

The partition coefficient P', expressed as a mole-fraction of substrate between lipid and water, can be obtained from

$$P' = \frac{55.6}{1.33} P = 41.8\, P \tag{11}$$

assuming a mean molecular mass for membrane phospholipids of 750 kDa. Likewise, the K_m of the enzyme can calso be expressed as the mole fraction of substrate in the membrane lipids as

$$K''_m = 1.33\, K'_m \tag{12}$$

If the substrate molecules interact with the enzyme-active site from within the lipid bilayer, addition of phospholipids to the assay medium increases the value of V_L and decreases the concentration in the lipid phase, resulting in the observation that the K_{app} increases with increasing the concentration of total lipid in the assay medium.

It should be noted that the demonstration of a decrease in K_{app} with increasing the concentration of lipid is not, by itself, evidence for the substrate interacting from the lipid phase. An equation analogous to Eqn. 10, viz.

$$K_{app} = \alpha(K'_m P - K'_m) + K'_m \tag{13}$$

can be derived on the assumption that the substrate reacts from the aqueous phase. This alternative equation also requires that K_{app} increases with increasing lipid. Because the volume fraction of water decreases on adding phospholipid, K_{app} will be greater than K'_m though the difference between the observed and intrinsic values will be small (the volume fraction of water changes very little with addition of lipid). However, the value of P obtained will be independent of either assumption used in analyzing the data.

Results

Ubiquinol cytochrome *c* reductase (E.C. 1.10.2.2) catalyzes the following reaction:

$$QH_2 + 2\,\text{Cyt } c(\text{ox}) \rightarrow Q + 2\,\text{Cyt } c(\text{red}) + 2H^+$$

where the reducing substrate ubiquinol (QH_2) is lipid soluble (ubiquinol-10 in bovine heart mitochondria); the enzyme is usually assayed using short chain ubiquinol homologs (ubiquinol-1 or ubiquinol-2) or analogs [10].

Fig. 1a shows double reciprocal plots of ubiquinol cytochrome *c* reductase activity at different α, obtained by addition of different amounts of asolectin vesicles to submitochondrial particles, and using ubiquinol-1 as the variable substrate and a concentration of cytochrome *c* sufficient to saturate the enzyme under all conditions, to avoid complications due to differential cytochrome *c* binding to the membrane and to approximate the single substrate Michaelis-Menten expression. The plots extrapolate to the same V_{max} but with the apparent K_m, K_{app}, strongly increasing with increasing α. This phenomenological behavior is strong evidence that the quinol substrate must partition between the water and the lipid phase, but, as pointed out in the Theory section, it cannot identify from which phase the substrate reacts with the active site of the enzyme.

TABLE I

KINETIC CONSTANTS OF UBIQUINOL CYTOCHROME *c* REDUCTASE AND PARTITION COEFFICIENTS OF UBIQUINOLS IN SUBMITOCHONDRIAL PARTICLES

	Duro-quinol	Ubiquinol-1	Ubiquinol-2
K_{app} (no addition) (μM) [a]	176	17	3.2
K'_m (mM in the lipid bilayer)	–	16	20
V_{max} (μmol·min^{-1}·mg^{-1})	0.50	3.4	15.2
k_{cat} (s^{-1}) [b]	42	283	1270
k_{cat}/K'_m (M^{-1}·s^{-1})	–	$1.8 \cdot 10^4$	$6.3 \cdot 10^4$
P (molar units)	–	$9.2 \cdot 10^2$	$2.0 \cdot 10^4$
P (mole fraction)	–	$3.8 \cdot 10^4$	$8.5 \cdot 10^5$

[a] Under the conditions employed (at saturating cytochrome *c* concentrations) the Lineweaver–Burk plots were linear and only one apparent K_m was extrapolated, contrary to what found at low cytochrome *c* concentrations [4,10,22].

[b] Calculated from the concentration of cytochrome c_1 in submitochondrial particles (0.21 nmol/mg protein).

Although the lower homologs of Q_{10} are much more soluble in water, it is most likely that they react at the same site as the physiological substrate which, because of its extreme hydrophobicity, undoubtedly reacts from the lipid phase. In particular, it is known that the kinetic mechanism [12], the sensitivity to inhibitors [13], the variation in activity with temperature [14,15] and the capacity to support phosphorylation and proton uptake [16] are essentially similar with lower and higher Q homologs, making it quite unlikely that there are different sites of reaction for ubiquinol-1 and ubiquinol-10. Consequently, we believe that the lower homologs also react from the lipid phase and have examined the effect of the addition of phospholipids on the steady-state kinetics using this assumption.

A replot of K_{app} vs α (Fig. 1, inset) gives a straight line: using Eqn. 10, the slope yields a K'_m of 16 mM, and P for ubiquinol-1 obtained from the intercept on the ordinate is $9.2 \cdot 10^2$ in molar units [(mol QH_2/litre PL): (mol QH_2/litre water)], corresponding to $3.8 \cdot 10^4$ in mole fraction units [(mol QH_2/mol PL): (mol QH_2/mol water)].

The same experiment using ubiquinol-2 as substrate yielded a K'_m of 20 mM and $P = 8.5 \cdot 10^5$ (mole fraction units).

It must be noted that quite different K_{app} (17 μM for ubiquinol-1 and 3.2 μM for ubiquinol-2) in submitochondrial particles correspond to almost identical K'_m (16 and 20 mM) in the phospholipid phase, a consequence of the different partition coefficients.

The V_{max} from the plots are 3.4 μmol · min^{-1} ·

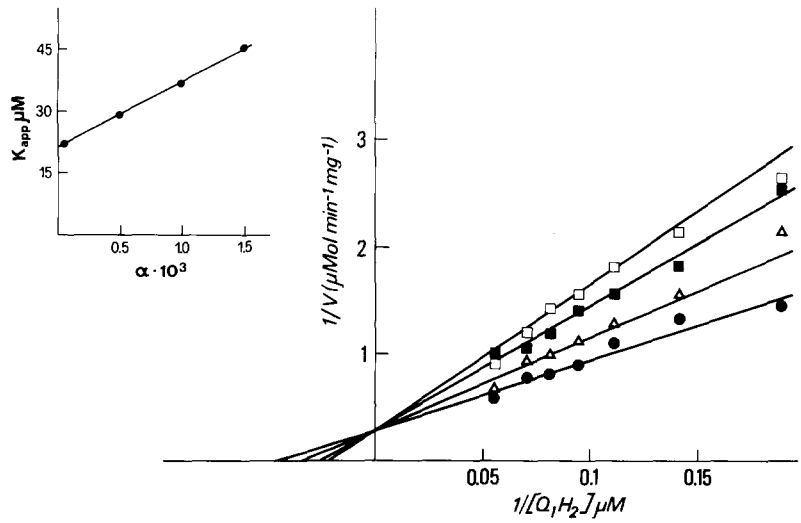

Fig. 1. Titration of ubiquinol cytochrome *c* reductase activity with ubiquinol-1 in submitochondrial particles (0.1 mg protein/ml) at different dilutions with exogenous liposomes. The total phospholipid concentrations ($\alpha \cdot 10^3$) were: ●———●, 0.05; △———△, 0.5; ■———■, 1.0; □———□, 1.5. In the inset is shown the replot of K_{app} vs. α. In the Lineweaver–Burk plots, the most suitable lines were drawn by inspection; the replot was calculated by least-squares regression analysis.

mg^{-1} for ubiquinol-1 and 15.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for ubiquinol-2. Since V_{max} for each ubiquinol is independent of α (Fig. 1a), it is clear that the catalytic constant k_{cat} ($= V_{max}/[E_T]$) increases with the increasing isoprenoid chain length of the ubiquinol homolog (cf. Table I).

Similar results were obtained by using a crude preparation of the bc_1 complex (fraction R_4B [5]) inlayed in phospholipid vesicles. Proteoliposome preparations obtained by cholate dialysis, having different lipid:protein ratios in the same vesicle, were assayed for ubiquinol-cytochrome *c* reductase activity, taking care that the protein content were the same in all assays, thus varying the phospholipid content per assay (and hence α). The residual detergent content was such that no respiratory control was apparent, and the reaction rates were not further enhanced by uncouplers. Fig. 2a and b shows double reciprocal plots at different α and the corresponding replots, using ubiquinol-2 as the variable substrate and saturating cytochrome *c*. The partition coefficients are very close to those found in submitochondrial particles ($P = 5 \cdot 10^5$ in mole fraction units), while K'_m is somewhat higher (81 mM in the phospholipids).

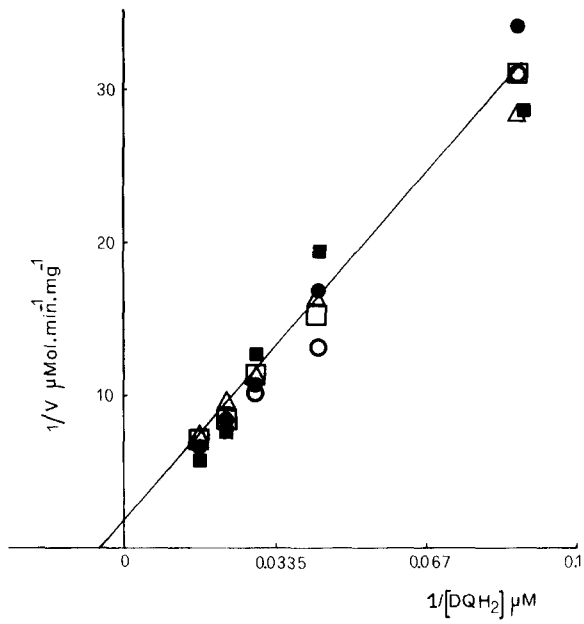

Fig. 3. Titration of ubiquinol cytochrome *c* reductase activity with duroquinol in submitochondrial particles (0.1 mg protein/ml) at different dilutions with exogenous liposomes. The total phospholipid concentrations ($\alpha \cdot 10^3$) were: ○——○, 0.5; △——△, 1.0; ●——●, 1.5; □——□, 2.0; ■——■, 2.5. The most suitable line was drawn by inspection.

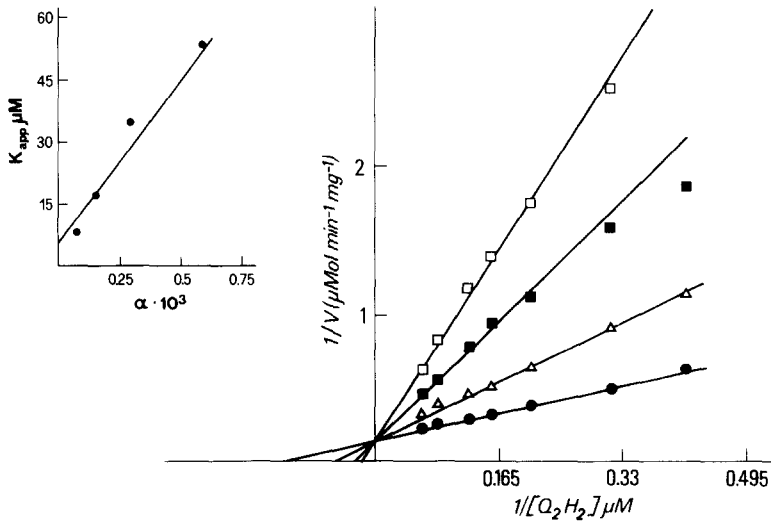

Fig. 2. Titration of ubiquinol cytochrome *c* reductase activity with ubiquinol-2 in mitochondrial fraction R_4B (7.33 μg protein/ml) inlayed in liposomes at different phospholipid contents obtained by cholate dialysis. The total phospholipid concentrations ($\alpha \cdot 10^3$) were: ●——●, 0.073; △——△, 0.147; ■——■, 0.293; □——□, 0.587. In the inset is shown the replot of K_{app} vs. α. In the Lineweaver–Burk plots the most suitable lines were drawn by inspection, while the replot was calculated by least-squares regression analysis.

We have also applied the method to the ubiquinol analog, duroquinol (2,3,5,6-tetramethyl benzoquinol). Duroquinone is very water-soluble; its partition coefficient in octanol/water has been determined to be 2 (in molar units) (Fato, R. and Lenaz, G., unpublished data); duroquinol, the analog of ubiquinol, is even more water-soluble; however, due to rapid autooxidation in water, we have not been able to determine its actual partition coefficient. Fig. 3 clearly shows that the double reciprocal plots of duroquinol cytochrome *c* reductase activity in submitochondrial particles are independent of the phospholipid concentration in the assay medium; such behavior is expected from both Eqns. 10 and 13, provided that the concentration in the lipid phase does not exceed that in the aqueous phase. If the concentration in the two phases is the same, $K_{app} = K'_m$, while if the solubility in the lipid phase is smaller then $K_{app} > K'_m$.

The method could not be applied to ubiquinol-3, because it aggregates so readily in water [17] that Michaelis–Menten behavior is not observed; even with this homolog, however, the apparent affinity of the enzyme appeared to decrease with increasing phospholipid concentration.

Discussion and Conclusions

The simplest way of determining the kinetic constants at varying lipid fractional volumes (see Theory) is to dilute the membrane enzyme with increasing amounts of pure lipid vesicles. If the rates expressing partition

$$QH_{2(W)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} QH_{2(L)}$$

are much greater than the enzymic reaction rates, the substrate concentration at any given time during the reaction course is substantially identical in the enzyme-free and the enzyme-containing membranes, and the whole lipid fractional volume can be treated as a homogeneous phase, in accordance with the theoretical premise. This seems to be the case for the shortest chain ubiquinol homologs, which form micelles in water only at relatively high concentrations (above those used for the kinetic assays) (cf. Refs. 17 and 18); accordingly, the quenching of fluorescence of membrane-bound fluorescent probes by short-chain ubiquinones is too fast to be resolved kinetically, indicating that the quinones partition into the membrane with a very fast k_1 (Fato, R. and Lenaz, G., unpublished results).

The partition coefficients obtained by this method in bovine heart submitochondrial particles can be compared with those found by other methods; the value obtained for ubiquinol-2 from the critical micelle concentration of the quinol in water [17,18] is about three times that obtained by the present method. The experimentally determined partition of a Q homolog in the lipid bilayer reflects the probability of the monomeric form added from ethanolic solution entering the membrane or forming a micellar aggregate in water [17,18]; whatever ubiquinol aggregate be present in the water phase would not be available as substrate to the enzyme-active site, if it reacts from within the lipid bilayer: the slightly lower values of the partition coefficient determined from the enzymic assay in comparison with the theoretical coefficient calculated from the critical micelle concentration indicates that under our experimental conditions only small amounts of micellar aggregates in water are formed.

The K'_m determined by this method can be compared with those calculated from K_{app} at a given lipid-to-water ratio, using the partition coefficients determined from the critical micelle concentration; the values determined with the two methods are usually in rather good agreement.

Since k_{cat} increases with the isoprenoid chain length of the ubiquinol homolog, whereas K'_m remains constant (Table I), it is also clear that the minimal second-order rate constant for ubiquinol oxidation, k_{cat}/K'_m, must also increase with increasing the isoprenoid chain length. It therefore appears likely that the catalytic rate, at least with ubiquinol-1, is reaction- and not diffusion-limited. Since diffusion coefficients and interaction molecular radii for different ubiquinone homologs are quite similar [17], were the rates diffusion-limited, the second-order rate constants for ubiquinol oxidation by the complex, calculated according to the Smoluchowski treatment [19], would be expected very similar for the different homologs. The K_{cat}/K'_m ratio shown in Table I for

ubiquinol-1 ($1.75 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 20°C) is not far from the second-order rate constant for ubiquinol-1 oxidation directly determined in the bc_1 complex isolated from yeast mitochondria [20], calculated taking in account the ubiquinol concentration in the residual lipid contained in the isolated complex (a value of $1.3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ in the lipids was calculated for 10 μM bc_1 complex containing 0.5 mg phospholipids per mg protein, and exhibiting an apparent first-order rate constant of 200 s^{-1} at 8°C, using 100 μM ubiquinol-1 [20]).

In conclusion, the method we have employed appears suitable for determination of partition coefficients, providing that little or no substrate forms micellar phases in the water solution, and the enzyme-catalytic constants are not affected by phospholipids, as seems to be the case for some membrane-bound enzymes [21]: in case of lipid dependence, however, the V_{max} extrapolated at different α would be different, and this has not been found for ubiquinol cytochrome *c* reductase under the experimental conditions of this report.

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