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DETERMINATION OF THE EQUILIBRIUM CONSTANT FOR THE BINDING OF FERRICYTOCHROME *c* TO PHOSPHOLIPID VESICLES AND THE EFFECT OF BINDING ON THE REDUCTION RATE OF CYTOCHROME *c*

JOHN B. CANNON and JAMES E. ERMAN

Department of Chemistry, Northern Illinois University, DeKalb, IL 60115 (U.S.A.)

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Summary

The rate of reduction of cytochrome *c* by 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was examined as a function of binding to liposomes prepared from mixed soybean phospholipids, asolectin, and from various purified phospholipids. Binding of cytochrome *c* to asolectin liposomes caused an increase in the rate of reduction by the pteridine derivative from 2900 to 16 000 $M^{-1} \cdot s^{-1}$ at pH 7. At low ionic strength (0.003 M) the binding stoichiometry between cytochrome *c* and asolectin vesicles is 15 ± 2 phosphospolipid/cytochrome *c* (mole ratio), determined by monitoring the change in reduction rate of cytochrome *c* by pteridine as cytochrome *c* is bound to the vesicles. A stoichiometry of 14 phospholipid/cytochrome *c* was obtained from gel filtration studies. Equilibrium association constants for the binding of cytochrome *c* to sites on the asolectin vesicles varied from $2.2 \cdot 10^6$ to $1.8 \cdot 10^3$ M^{-1} between 0.02 and 0.10 M ionic strength, respectively. In general, liposomes prepared from purified phospholipids resulted in less binding of cytochrome *c* per mole of phospholipid and lower reduction rates than those prepared from asolectin.

Introduction

The interaction between the cytochromes of the electron transport chain and the phospholipid bilayer of the inner mitochondrial membrane affects the reactivity and function of the cytochromes in ways which are not completely

Abbreviations: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

understood. The interaction can be primarily hydrophobic and intimate, as is the case for cytochromes *b*, c_1 and cytochrome oxidase, or primarily electrostatic and confined to surface interactions, as is the case for cytochrome *c* [1]. In an attempt to understand better the nature of the interactions and to determine the effect of the phospholipid bilayer on the reactivity of the cytochromes, we have begun a study of the effect of liposomes (phospholipid bilayer vesicles) on the reduction kinetics of ferricytochrome *c*. Cytochrome *c* has been previously shown to bind to the external surface of liposomes primarily by electrostatic attraction of the positively charged protein and negatively charged liposomes, as evidenced by the fact that high ionic strength disrupts the complexes [2,3]. To shed further light on the nature of this interaction, we wish to report a study of the effect of liposomes on the kinetics of reduction of ferricytochrome *c* by the neutral reductant, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$). We also report a kinetic method which allows measurement of binding constants of cytochrome *c* and liposomes as a function of ionic strength.

Materials and Methods

Horse heart cytochrome *c* (Sigma, Type VI) was used without further purification; concentrations were measured by the absorbance at 410 nm using an extinction coefficient of 106.1 $mM^{-1} \cdot cm^{-1}$. $DMPH_4$, purchased as the hydrochloride (Aldrich), was recrystallized from water/alcohol/acetone [4]; solutions of $DMPH_4$ were prepared by weight and neutralized with KOH under nitrogen. Suspensions of 1% soybean asolectin liposomes were prepared as described previously [5]. Asolectin was homogenized in water and vesicles prepared by sonication in a Branson sonifier (Model W-350) using an immersion tip. Sonication was carried out at 25—30°C until the suspension clarified, usually 30—45 min. The sonicated vesicles were centrifuged at 160 000 × *g* for 30 min and the supernatant dialyzed overnight against water prior to use. In some cases the crude asolectin was partially purified by dissolving the material in nitrogen-saturated chloroform and filtering. The filtrate was evaporated to dryness, and the residue was stirred with acetone at 4°C for 24 h. After filtration, the yellowish-white solid was collected and used for liposome preparation; this partial purification had no effect on the kinetic results.

The following phospholipids were purchased from Sigma and used without purification: dimyristoyl-L-α-phosphatidylcholine, 98%; dioleyl-L-α-phosphatidylcholine, 98% in chloroform; dipalmitoyl-L-α-phosphatidic acid, sodium salt; dipalmitoyl-L-α-phosphatidylethanolamine; L-α-phosphatidylethanolamine, Type IV from soybean, 98%, in chloroform; L-α-phosphatidylinositol, sodium salt, Grade I from soybean, 98%; cardiolipin, sodium salt, from bovine heart, in ethanol; oleyl-L-α-lysophosphatidylcholine. Liposomes were prepared from mixtures of these phospholipids by dissolving the appropriate weights of phospholipid in chloroform to insure homogeneity, evaporating to dryness with a rotatory evaporator, suspending in the required volume of water to make mixtures of 0.3—0.4% lipid, sonicating and centrifuging as previously described [5].

The phospholipid content of the liposome preparations was determined by a

phosphate analysis using a variation of the method of Fiske and Subbarow [6]. Visible spectra were taken on a Cary 219 spectrophotometer and CD spectra were recorded by a JASCO model ORD/UV-5 spectrometer. Gel filtration was performed using a Sephadex G-75 column, following the absorbance at 254 and 405 nm using an ISCO UA-5 absorbance monitor. Reduction of cytochrome *c* was followed at 550 nm (at 25°C) as previously described [5] using 1—10 mM potassium phosphate (pH 7) as the buffer with KNO_3 added to adjust the ionic strength.

Results

The rate of reduction of ferricytochrome *c* by $DMPH_4$ is independent of ionic strenth at pH 7, 25°C, with a value of $(2.9 \pm 0.4) \cdot 10^3\ M^{-1} \cdot s^{-1}$. Addition of asolectin vesicles to solutions of cytochrome *c* increases the reduction rate. Fig. 1 presents the results obtained at 0.003 M ionic strength. The apparent bimolecular rate constant for the reduction of cytochrome *c* by $DMPH_4$ increases linearly with increasing mole ratio of phospholipid : cytochrome *c* until a maximum value of $(1.6 \pm 0.2) \cdot 10^4\ M^{-1} \cdot s^{-1}$ is reached at a mol ratio of 15 ± 2. This mole ratio most likely represents the stoichiometry of binding of cytochrome *c* to the asolectin vesicles. This stoichiometry was confirmed by a gel filtration experiment shown in Fig. 2. Mixtures of various ratios of liposome to cytochrome *c* were applied to a Sephadex G-75 column. In these mixtures the phospholipid concentration was held constant at 0.50 mM and the amount of cytochrome *c* varied from 6 to 65 μM. The point at which free cytochrome *c* begins to appear represents the point of saturation of liposomes with cytochrome *c*. The stoichiometry of bindng obtained from the gel filtration study is a mol ratio of 14 phospholipid : cytochrome *c*, agreeing well with the kinetic result.

Fig. 3 presents the results of the kinetic titration experiment at several ionic strength values. As the ionic strength is increased the binding of cytochrome *c* to the liposomes decreases and the titration curves show pronounced departure

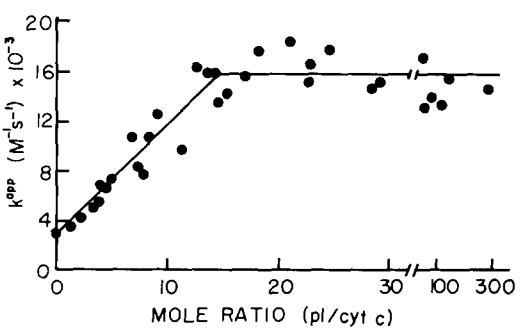

Fig. 1. Apparent bimolecular rate constant for the reduction of ferricytochrome *c* by $DMPH_4$ as a function of asolectin phospholipid : cytochrome *c* mole ratio (pl/cyt c). All experiments were performed at an ionic strength of 0.003 M, phosphate buffer, pH 7, at 25°C. Cytochrome *c* concentrations varied between 0.8 and 3.2 μM. $DMPH_4$ concentrations varied between $4 \cdot 10^{-5}$ and $1.6 \cdot 10^{-3}$ M. The apparent bimolecular rate constant was independent of $DMPH_4$ concentration.

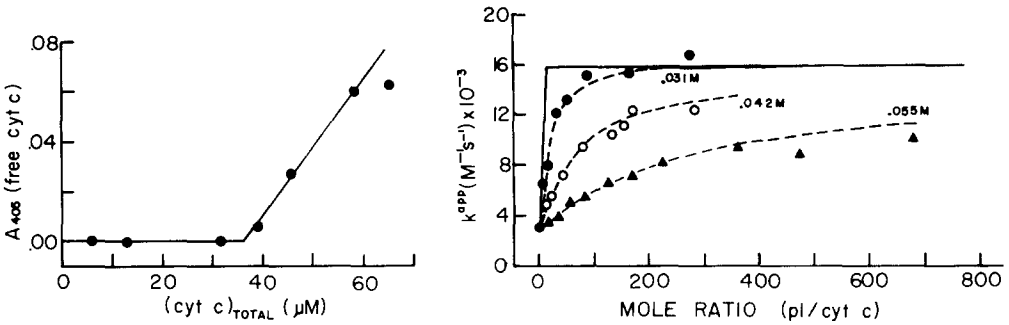

Fig. 2. Gel filtration on Sephadex G-75 of cytochrome/asolectin vesicle mixtures. Asolectin phospholipid concentration was held constant at 0.501 mM. The absorbance in the free cytochrome *c* (unbound) peak is plotted as a function of the total cytochrome *c* added to the asolectin vesicles. All reactions were carried out at an ionic strength of 0.003 M, in phosphate buffer, pH 7, at ambient temperature.

Fig. 3. Apparent bimolecular rate constant for the reduction of ferricytochrome *c* by $DMPH_4$ as a function of asolectin phospholipid : cytochrome *c* mole ratio at the ionic strength indicated. The solid line indicates stoichiometric binding determined from the data in Fig. 1. The dashed lines are calculated based on the equilibrium binding model discussed in the text. The cytochrome *c* concentration was 1.6 μM and all reaction conditions except ionic strength are analogous to those in Fig. 1.

from the stoichiometric binding curve. The kinetic titration curves shown in Fig. 3 can be evaluated using the mechanism shown in Eqn. 1 below.

$$\begin{array}{ccc} & \text{C(III)} & \xrightarrow{k_1\,(\text{reductant})} & \text{C(II)} \\ K_A \;\Big\uparrow\Big\downarrow & +\,V & & \Big\uparrow\Big\downarrow\; +\,V \\ & \text{C(III)}\cdot V & \xrightarrow{k_2\,(\text{reductant})} & \text{C(II)}\cdot\cdot V \end{array} \qquad (1)$$

In the scheme shown in Eqn. 1, C(III) and C(II) represent free ferri- and ferrocytochrome *c*, respectively; C(III) · V and C(II) · V represent ferri- and ferrocytochrome bound to phospholipid vesicles, k_1 and k_2 are the rate constants for reduction of free and bound ferricytochrome *c*, and K_A is the equilibrium association constant for the binding of ferricytochrome *c* to the phospholipid vesicles. Under the experimental conditions employed, the reduction of ferricytochrome *c* was always monophasic, indicating that the equilibration of free and bound cytochrome *c* is rapid compared to the reduction rate (greater than 29 s^{-1} [5]). Using these considerations, the apparent bimolecular rate constant for the reduction of ferricytochrome *c* is given by Eqns. 2 and 3:

$$k^{app} = k_1 - \frac{(k_1 - k_2)}{2C_t}[b - (b^2 - 4C_t N V_t)^{1/2}] \qquad (2)$$

where

$$b = C_t + NV_t + \frac{1}{K_A} \qquad (3)$$

In Eqns. 2 and 3, k_1, k_2 and K_A are defined in Eqn. 1, C_t and V_t represent the total molar concentration of ferricytochrome *c* and phospholipid, respectively,

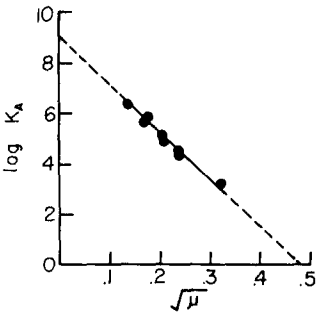

Fig. 4. A plot of the logarithm of the equilibrium association constant for the binding of ferricytochrome *c* to asolectin vesicles as a function of the square root of ionic strength at pH 7, 25°C.

and N represents the stoichiometry of the cytochrome *c* binding sites on the phospholipid vesicles.

It has been shown that k_1 is independent of ionic strength at pH 7 under the conditions employed in this study [5]. Between 0.003 and 0.031 M ionic strength, cytochrome *c* can be completely bound to asolectin vesicles and k_2 can be evaluated directly. Between 0.04 and 0.06 M ionic strength, we have not achieved complete binding of cytochrome *c* to the vesicles, but double-reciprocal plots show that the apparent bimolecular rate constant extrapolates to a value of $1.6 \cdot 10^4\ M^{-1} \cdot s^{-1}$, within experimental error, at infinite concentrations of phospholipid. We conclude that k_2 is independent of ionic strength. While it seems probable that there are several types of binding site for cytochrome *c* on vesicles made from asolectin, since asolectin is a complex mixture of various types of phospholipids, the simple mechanism shown in Eqn. 1, implying only a single type of binding site with an equilibrium constant, K_A, is adequate to fit our experimental data. A non-linear least-squares curve-fitting program [7] was used to evaluate K_A, using Eqn. 2, from the titration curves obtained at various ionic strengths. The values of k_1, k_2 and N were held constant during the fitting procedure. Values of K_A vary with ionic strength and a plot of log K_A as a function of the square root of ionic strength is shown in Fig. 4. The dashed lines in Fig. 3 were calculated using the best-fit values of K_A and Eqn. 2.

In order to investigate the reason for the higher reactivity of bound cytochrome *c* toward $DMPH_4$ and also to examine the effect of phospholipid composition on the kinetic behavior, cytochrome *c* reduction rates were measured in the presence of liposomes made from various purified phospholipid mixtures. Table I gives a summary of experiments performed at an ionic strength of 0.003 M and shows a comparison of k_2, which represents the rate constant of reduction of liposome-bound cytochrome *c* by $DMPH_4$, and the stoichiometry, determined from kinetic titration experiments analogous to that shown in Fig. 1 as a function of liposome composition. The stoichiometries of cytochrome *c* binding could not be determined for all liposomes because of turbidity at liposome : cytochrome *c* ratios near the equivalence point. In all cases, the solutions remained clear at high vesicle-to-cytochrome *c* ratios. While liposomes prepared from purified phospholipids always gave rates at least twice

TABLE I

DEPENDENCE OF CYTOCHROME *c* REDUCTION BY PTERIDINE AND BINDING STOICHIOMETRIES ON LIPOSOME COMPOSITION

Conditions: 0.003 M ionic strength, pH 7, phosphate buffer, 25°C. DMPC, dimyristoyl phosphatidylcholine; DPPA, dipalmitoyl phosphatidic acid; SoyPI, soybean phosphatidylinositol; DOPC, dioleyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; SoyPE, soybean phosphatidylethanolamine; C1, cardiolipin; LyPC, oleyl lysophosphatidylcholine. Stoichiometry expressed as mol phospholipid/mol cytochrome *c*.

Liposome composition	Wt. ratio	$k_2 \times 10^{-3}$ ($M^{-1} \cdot s^{-1}$)	Stoichiometry
Asolectin	—	16 ± 2	15 ± 2
DMPC/DPPA	4/1	5.0 ± 0.5	35 ± 10
DMPC/DPPA	2/1	4.9 ± 1.0	37 ± 12
DMPC/SoyPI	3/1	5.2 ± 0.8	39 ± 7
DOPC/DPPA	4/1	5.5 ± 0.5	43 ± 6
DMPC/DPPE/DPPA	3.3/2.3/1	5.2 ± 1.4	turbid
DOPC/SoyPE/DPPA	3/1/1	5.9 ± 0.3	54 ± 5
DOPC/SoyPE/DPPA	1/3/1	8.6 ± 1.6	turbid
DOPC/SoyPE/SoyPI	2.2/1.7/1	6.9 ± 0.6	turbid
DOPC/SoyPE/SoyPI	1.7/2.2/1	6.8 ± 0.8	turbid
DOPC/SoyPE/SoyPI/C1/LyPC	3.3/4.4/1/1/1	8.2 ± 0.6	50 ± 13

that of free cytochrome *c*, these liposomes gave significantly lower rates and higher binding stoichiometries than asolectin liposomes.

Discussion

The mechanism for reduction of cytochrome *c* illustrated in Eqn. 1 applies to both ascorbate [5] and $DMPH_4$; the observed rates are average rates based on the relative contributions from free and bound cytochrome *c*. For ascorbate, k_1 is greater than k_2 and the values of both are dependent upon ionic strength. For the neutral $DMPH_4$, k_2 is greater than k_1 and both are independent of ionic strength. The absence of electrostatic effects between $DMPH_4$ and cytochrome *c* or liposomes allows isolation of the electrostatic interaction between cytochrome *c* and liposomes.

The experimental value for the stoichiometry of cytochrome *c* binding to asolectin vesicles is a mol ratio of 15 ± 2 phospholipid : cytochrome *c*. From the average dimensions of sonicated liposomes, with an outer diameter of 250—300 Å, and an inner aqueous core diameter of 130—170 Å [8], it is apparent that only about 75% of the phospholipids of such a liposome are on the surface and available for binding cytochrome *c*. This means that a single cytochrome *c* binding site of the asolectin liposome surface would contain about 12 ± 2 phospholipid molecules. From the dimensions of cytochrome *c* [9], the minimum area a bound cytochrome *c* molecule would occupy is about 1050 $Å^2$ while that of a typical phospholipid molecule (phosphatidylcholine) has been calculated to be 75 $Å^2$ [10]. Thus, in theory, one would expect a minimum value of 1050 Å/75 Å or 14 mol of phospholipid/mol cytochrome *c* as the binding site stoichiometry. This comparison of experimental and theoretical values indicates that there is close packing of the cytochrome *c* molecules on the asolectin liposome surface in the fully bound state. The fact

that the experimental value is slightly smaller than the minimum theoretical value could be due to experimental error, to the possibility that the asolectin could contain some lipids other than phospholipid, and to the approximate nature of the theoretical value including treating both the cytochrome *c* and the liposome as rigid particles. It may also be possible that the binding of cytochrome *c* causes the phospholipid in the bilayer to 'spread out', increasing the surface area available for binding. Such a phenomenon has been suggested from measurements of surface pressures of cytochrome *c*/phospholipid monolayer systems [11]. As seen in Table I, liposomes prepared from purified phospholipid mixtures generally show stoichiometries at least twice as large in terms of mole ratios of phospholipid/cytochrome *c* as does asolectin. Cytochrome *c* does not pack as tightly on the liposomes prepared from purified phospholipids as it does on asolectin vesicles. Although one would expect that the binding of cytochrome *c* to phospholipid vesicles would be influenced by the ratio of acidic (negatively charged) phospholipid to total phospholipid in the vesicles, the effect is not apparent over the limited range of composition used in this study. Asolectin has a maximum of 25—27% acidic phospholipids (based on phosphate analysis), mainly cardiolipin and phosphatidylinositol [12,13], while the synthetic liposomes have between 20 and 33% acidic phospholipid. The gross percentage of acidic phospholipid cannot explain the differences between asolectin vesicles and synthetic vesicles in terms of their interaction with cytochrome *c*.

The plot of the equilibrium association constant as a function of the square root of ionic strength (Fig. 4) can be qualitatively evaluated using the limiting Debye-Huckel theory (Eqn. 4):

$$\log K_A = \log K_A^0 + 1.02\, Z_v Z_c \sqrt{\mu} \qquad (4)$$

The value of the equilibrium constant extrapolated to zero ionic strength, K_A^0, is $(1.0 \pm 0.2) \cdot 10^9\ M^{-1}$ and the slope is -19 ± 2. Assuming that Z_c represents the net charge on cytochrome *c*, which is +6.0 at pH 7 [14], a value of −3.1 is calculated for Z_v from the slope of the plot in Fig. 4. This represents the effective charge of the cytochrome *c* binding site on the surface of asolectin vesicles and agrees well with the value of −3 calculated from the stoichiometry of cytochrome *c* binding and the phospholipid composition of asolectin [12,13].

In conclusion, the kinetics of reduction of ferricytochrome *c* by $DMPH_4$ represent a convenient and simple system with which to monitor the binding of cytochrome *c* to liposomes. It leads to a convenient kinetic method to measure the binding stoichiometries and binding constants over a relatively wide range of ionic strength. The rate of equilibration of cytochrome *c* with the liposome binding sites is fast compared to the reduction rates under the conditions employed in this study.

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