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THE EFFECT OF PHOSPHOLIPID VESICLES ON THE KINETICS OF REDUCTION OF CYTOCHROME C

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SUMMARY

The rate of reduction of cytochrome c by ascorbate and by 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was examined as a function of ionic strength and of binding to phospholipid vesicles (liposomes). Binding of cytochrome c to liposomes, which occurs at low ionic strength, decreases the rate of reduction by ascorbate by a factor of up to 100, which can be primarily explained on electrostatic grounds. In the absence of liposomes, kinetics of reduction by the neutral pteridine derivative showed no ionic strength dependence. Binding of cytochrome c to liposomes increased the rate of reduction by pteridine. An estimation of the binding constant of cytochrome c to liposomes at 0.06 M ionic strength, pH 7, is given.

Cytochrome c, an important component of the electron transport chain, is present between the outer and inner mitochondrial membranes. The effect of the membrane on its reactivity is, however, not yet understood (1). The binding of cytochrome c to the external surface of phospholipid vesicles (liposomes) provides a good model system for the examination of this question. Most workers in this field have shown that the nature of the interaction of cytochrome c with such liposomes is predominantly electrostatic, i.e., a charge attraction between the positively charged cytochrome c and the negatively charged liposomes. High ionic strength disrupts the complexes (2,3). There is some evidence, however, that the interaction is partly hydrophobic, that there is partial penetration of the cytochrome c molecule into the lipid bilayer (4-6). We report herein an examination of the effect of liposome binding on the kinetics of horse heart cytochrome c reduction by the negatively charged

Abbreviations: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine; Tris, Tris(hydroxymethyl)aminomethane; Pi, phosphate.

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reductant ascorbate, and also the neutral reductant $DMPH_4$. A study of ionic strength dependence revealed behavior for the reduction by $DMPH_4$ that cannot be explained purely by electrostatic binding.

MATERIALS AND METHODS

Fresh horse heart cytochrome c (Sigma, Type VI) was used without further purification. Solutions of ascorbic acid (Fisher) were neutralized with KOH and the concentration determined by iodometric titration (7). $DMPH_4$ was purchased as the hydrochloride (Aldrich); solutions of $DMPH_4$ were prepared by weight and neutralized with KOH under nitrogen. Phospholipid vesicles were prepared by homogenizing the crude soybean Asolectin (Associated Concentrates) in water, sonicating with a Branson Sonifier for 45 minutes, and centrifuging at 160,000 g for 30 minutes. The slightly turbid supernatant which was used for the experiments could be analyzed by a variation of the Fiske-Subbarow method (8), and generally showed phospholipid content corresponding to 70% of the original weight of asolectin. Kinetics with ascorbate were performed on a Cary 14 spectrophotometer, by observing the increase in absorbance at 417 nm. Kinetics with $DMPH_4$ were performed on a Durrum-Gibson Stopped Flow apparatus with observation at 550 nm. All solutions were deaerated by bubbling with nitrogen immediately before use.

RESULTS

Figure 1A shows the rate of reduction of cytochrome c by ascorbate in the presence and absence of vesicles as a function of ionic strength, in K-Pi/ KNO_3 (pH 7). A decrease in rate in the absence of vesicles is observed as ionic strength is increased, consistent with Debye-Hückel theory for a reaction involving the negatively charged ascorbate and the positively charged cytochrome c. Results in Tris-Cl (pH 8.0), Tris-cacodylate (pH 7.2), and potassium phosphate (pH 7) were qualitatively similar, although rates in Tris-Cl were faster than in any of the buffer systems. The results are in good agreement with rate constants previously reported; Table I gives a summary. Under our conditions and methods of measurement, kinetics were monophasic for >90% of the reaction; biphasic kinetics were never observed, in contrast to previous reports (9,10).

The reduction of cytochrome c by ascorbate in the presence of phospholipid vesicles (Figure 1A) was up to 100 times slower at low ionic strength; previous workers report a 15 fold decrease (11). Because the composition of asolectin (12) results in a negative charge on the vesicles at pH 7, the cytochrome c-

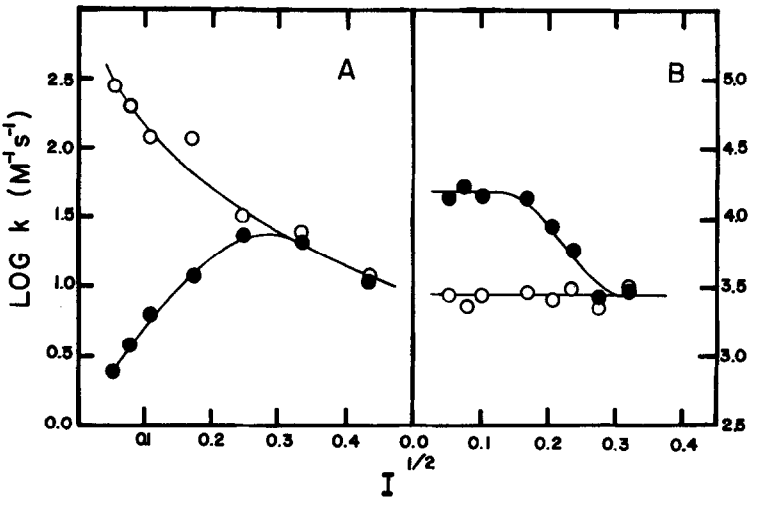

FIGURE 1: Reduction kinetics of cytochrome c as function of ionic strength, in 1-10 mM KPi (pH 7) buffer; KNO_3 was added to bring to required ionic strength. A: Cytochrome c (6 μM) + ascorbate.
Open Circles: [vesicles] = 0, [ascorbate] = 0.3 mM - 2 mM.
Solid Circles: [vesicles] = .7 mg/ml, [ascorbate] = 2 mM - 10 mM.
B: Cytochrome c (1.6 μM) + $DMPH_4$.
Open Circles: [vesicles] = 0, [$DMPH_4$] = 0.04 mM - 1.5 mM.
Solid Circles: [vesicles] = 0.18 mg/ml, [$DMPH_4$] = 0.02 mM - 1 mM.

TABLE I. Rate Constants for Reduction of Cytochrome c by Ascorbate

Conditions	k, M^{-1} s^{-1} (this work, without vesicles)	k, M^{-1} s^{-1}* (reported, without vesicles)	k, M^{-1} s^{-1} (this work with vesicles)
0.002 M phosphate, pH 7.0	300	--	3.6
0.01 M phosphate, pH 7.0	49	40-50 (9)	32
0.01 M Tris Cl, pH 8.0	1300	--	10.2
0.10 M Tris Cl, pH 8.0	450	750 (9)	160
0.002 M Tris cacodylate, pH 7.2	610	600 (10)	3.3
0.10 M Tris cacodylate, pH 7.2	64	--	45

*References given in parentheses.

vesicle complex would have a lower rate of reaction with the negatively charged ascorbate than would the more positively charged "free" cytochrome c. As ionic strength is increased to >0.15 M, the rates become equal to those obtained in the absence of vesicles, since high ionic strength disrupts the binding of cytochrome c to phospholipid vesicles (2,3). At no point were

biphasic kinetics observed for the reduction of cytochrome c in the presence of vesicles.

Because of the large electrostatic effect on the behavior of the cytochrome c-vesicle-ascorbate system, it was thought that a more fruitful area for examination might be the kinetics of reduction by $DMPH_4$ which is uncharged at pH 7 (13) As shown in Figure 1B, the rate of reduction of cytochrome c by $DMPH_4$ in the absence of vesicles is rapid and efficient, with a rate constant of $2.8 \pm 0.3 \times 10^3\ M^{-1}\ s^{-1}$ over a range of $DMPH_4$ concentration from 0.02 mM to 1.5 mM, and is independent of ionic strength. Figure 1B also shows the rate constant in the presence of phospholipid vesicles as a function of ionic strength. In contrast to ascorbate, at low ionic strength (μ = 0.003-0.03 M) this rate is 5 times faster than in the absence of vesicles. At high ionic strength (> .1 M), at which cytochrome c is not bound to vesicles, the rate is the same as that in the absence of vesicles. Between 0.03 M and 0.1 M ionic strength, intermediate rate values are observed, apparently due to the reaction of both bound and unbound cytochrome c. At no ionic strength was a biphasic reaction observed.

DISCUSSION

Other than specific ion effects (14), the results involving the cytochrome c-vesicle-ascorbate system can be understood primarily on electrostatic grounds. Below 0.1 M ionic strength, cytochrome c-vesicle complexes can form, which have a slower rate of reaction with ascorbate because of charge repulsions. The rates observed in the ionic strength range 0.03 M-0.1 M depend upon the relative amounts of vesicle bound and free cytochrome c which are present under these conditions. The fact that biphasic kinetics were not observed means that there is a rapid equilibrium between bound and free cytochrome c, and that the rate of dissociation of the cytochrome c-vesicle complex is faster than the observed reduction rate. Below 0.03 M ionic strength, cytochrome c should be present entirely in the bound form (as indicated by the $DMPH_4$ results). The rate continues to decrease as ionic strength is decreased below 0.03 M, because of Debye-Hückel effects. Since ionic strength affects

the binding of cytochrome c to the vesicles and the reduction rates of both bound and free cytochrome c, it is difficult to isolate each effect.

For the reductant $DMPH_4$, however, the actual reduction rates of bound and free cytochrome c are independent of ionic strength. At either low or high ionic strength, the observed rates of reduction reflect the reaction of either the completely bound form (below 0.03 M, $k = 1.54 \pm 0.14 \times 10^4\ M^{-1}\ s^{-1}$) or as the completely free form (above 0.1 M, $k = 2.8 \pm 0.3 \times 10^3\ M^{-1}\ s^{-1}$). Between 0.03 M and 0.1 M ionic strength, the fact that only monophasic kinetics were observed again means that there is rapid equilibrium between the bound and free cytochrome c. At 0.06 M ionic strength (at which $k = 5800\ s^{-1}$), the rate was first order in $DMPH_4$ up to a concentration of 5 mM, at which the observed rate is $29\ s^{-1}$. This value provides a lower limit for the dissociation rate of cytochrome c from the vesicles binding sites.

The simplicity of the kinetics of the $DMPH_4$-cytochrome c-vesicle system makes it possible to determine the proportion of bound and free cytochrome c at any ionic strength, equation 1:

$$\% \text{ free c} = \frac{k_{bound} - k_{obs}}{k_{bound} - k_{free}} \times 100 \qquad (1)$$

Using equation 1, one can calculate that there is 76% free cytochrome c at 0.06 M ionic strength. These calculations make possible an estimation of the equilibrium (dissociation) constant of cytochrome c-vesicle binding, equation 2:

$$K_D = \frac{[c]_f\ [v]_f}{[c\text{-}v]} \qquad (2)$$

where $[c]_f$ is the concentration of free cytochrome c, $[v]_f$ is the concentration of free vesicle binding sites, and [c-v] is the concentration of bound (occupied) vesicle binding sites. While accurate values of $[c]_f$ and [c-v] can be obtained, only a range of values can presently be obtained for $[v]_f$. The minimum concentration of total binding sites is the same as the total cytochrome c concentration, is this case, 1.6×10^{-6} M. The maximum concentration of total binding sites can be estimated as follows: since a single phospholipid molecule occupies a surface area of 72 Å^2 in a membrane (15), a phospho-

lipid concentration of 2.02×10^{-4} M (0.18 mg/ml) would offer 4.4×10^{21} $\mathring{A}^2$ total external surface area per liter for unilamellar bilayer vesicles. From the dimensions of cytochrome c (16), a single binding site would require a minimum of 1050 $\mathring{A}^2$, thus giving a maximum number of 4.2×10^{18} binding sites per liter or 6.9×10^{-6} M. At 0.06 M ionic strength, a value of 4.8 μM for K_D is obtained using the minimum estimate of the concentration of binding sites, while a value of 20 μM is obtained using the maximum estimate for binding site concentration. Although these limits for the dissociation constant for the binding of cytochrome c are fairly close, we are in the process of experimentally determining the concentration of binding sites in asolectin vesicles for a more accurate determination of K_D.

One of the most interesting and unexpected findings of these experiments was that the rate of reduction by $DMPH_4$ was faster for vesicle bound cytochrome c than for free cytochrome c. This could mean that binding to vesicles alters the conformation of ferricytochrome c, making it more susceptible to reduction by $DMPH_4$, e.g. by opening up the heme crevice. This lends support to the concept that cytochrome c binding to phospholipid vesicles involves hydrophobic interactions as well as electrostatic. It has been suggested that interaction with phospholipid weakens the heme crevice (17). Further work must be done to determine the reason for the effect of vesicle binding on the kinetics of $DMPH_4$ reduction of cytochrome c.

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