Thermodynamic Parameters for the Reduction Reaction of Membrane-Bound Cytochrome *c* in Comparison with Those of the Membrane-Free Form: Spectropotentiostatic Determination with Use of an Optically Transparent Thin-Layer Electrode†

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ABSTRACT: An optically transparent thin-layer electrode cell with a very small volume has been developed to investigate the formal reduction potential ($E^{0'}$) of horse heart cytochrome *c* with and without cardiolipin-containing lecithin liposomes. Temperature-dependent experiments of membrane-free cytochrome *c* (10–40 °C) yielded standard thermodynamic parameters in agreement with literature values. On the other hand, membrane-bound cytochrome *c* had a break point(s) at the range between 10 and 30 °C when the formal reduction potential was displayed with respect to temperature. Both enthalpy and entropy changes had large negative values in the high-temperature region (>30 °C) relative to those of the membrane-free form, suggesting membrane participation during the reduction course of cytochrome *c*. In addition, we observed an extrathermodynamic relationship between enthalpy and entropy values among different cytochrome *c*'s and horse heart cytochrome *c* with and without membrane.

In mitochondria, cytochrome *c*, which is attached to the surface of the inner membrane, mediates the electron transfer between cytochrome b–c_1 complex and cytochrome oxidase (Keilin & Hartree, 1938; Okunuki & Yakushiji, 1941). The function of membrane in the electron-transfer reaction has been implicated to be a proper orientation of the cytochrome *c* molecule relative to the oxidase and the b–c_1 complex, both of which are tightly bound to the membrane. In the literature, there are many works on the measurements of the reduction potential of horse heart cytochrome *c* (Dutton et al., 1970; Eddowes & Hill, 1979; Erecińska & Vanderkooi, 1975; Heineman et al., 1975; Kimelberg & Lee, 1970; Margalit & Schejter, 1970; Taniguchi et al., 1980; Vanderkooi et al., 1973b), including the thermodynamic parameters. About a decade ago, pioneer works were carried out for the membrane-bound form by Dutton et al. (1970), Kimelberg & Lee (1970), and Vanderkooi et al. (1973a,b). However, the thermodynamic parameters of the reduction reaction of membrane-bound cytochrome *c* has been reported only by Erecińska & Vanderkooi (1975), who used phosphatidylinositol vesicles. In view of the important role of membrane on the mitochondrial electron-transfer reaction, we decided to use a lecithin–cardiolipin membrane system to measure the reduction potential of artificial membrane-bound cytochrome *c* in comparison with the free form. We utilized an optically transparent thin-layer electrode, coupled with optical spectroscopy, to determine the potential. Our present paper describes a biphasic profile of reduction potential vs. temperature, revealing large negative values of both $\Delta H°$ and $\Delta S°$ above 30 °C, and the enthalpy–entropy compensation effect of various cytochrome *c*'s.

Materials and Methods

Horse heart cytochrome *c* (type VI), egg yolk lecithin (type V-E), dipalmitoylphosphatidylcholine, Tris,[1] and DCPIP were obtained from Sigma. Bovine heart cardiolipin, bovine liver phosphatidylinositol, and soybean phosphatidylinositol were from Avanti. EDTA was from Aldrich.

Cardiolipin–Lecithin Liposomes. Mixtures of egg lecithin and cardiolipin in the weight ratio of 4:1 were maintained at 35–40 °C in a water bath. A stream of nitrogen was flushed over the top of solution to evaporate the solvent. The dried lipids were redissolved in 2 mL of dry ether and redried. The mixture was then mechanically dispersed in a buffer of 10 mM Tris–1 mM EDTA, pH 7.0, at room temperature. The turbid solution was then sonicated by a Branson cell disruptor. Sonication was carried out at 0 °C for 10 min with a cooling period between each 2-min sonication. Nitrogen was flushed above the solution throughout the sonication procedure. The clarified solution was then centrifuged at 80000*g* for 10 min at 10 °C. The supernatant fluid was collected as the liposome sample. In case of cardiolipin–dipalmitoylphosphatidylcholine, sonication was performed at 40 °C.

Cytochrome c/Cardiolipin–Lecithin Complexes. The liposomes were first saturated with argon, and an appropriate amount of cytochrome *c*, dissolved in a minimal amount of buffer, was then added dropwise with stirring. The mixture was transferred to an Amicon pressure dialysis apparatus equipped with YM-30 ultrafilter paper. The concentrated solution was then carefully removed from the apparatus, transferred to an amber glass vial under argon atmosphere, and sealed with a rubber-septum cap. An appropriate aliquot was withdrawn with a Hamilton microsyringe for each experiment. A NaCl solution was then added to make a desired final NaCl concentration.

Cytochrome *c* was determined by reduced minus oxidized difference spectra with an extinction coefficient $\Delta\epsilon_{550-540}$ as 19 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959). For membrane-bound cytochrome *c* samples, the sample cuvette was placed at the very front of an end-on photomultiplier to minimize the light scattering. For calculation of a ratio of

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DCPIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid disodium salt; $E^{0'}$, formal reduction potential in millivolts vs. NHE; NHE, normal hydrogen electrode; SCE, saturated calomel electrode; E_{appld}, potential applied across SCE and working electrode; *n*, equivalent per reaction.

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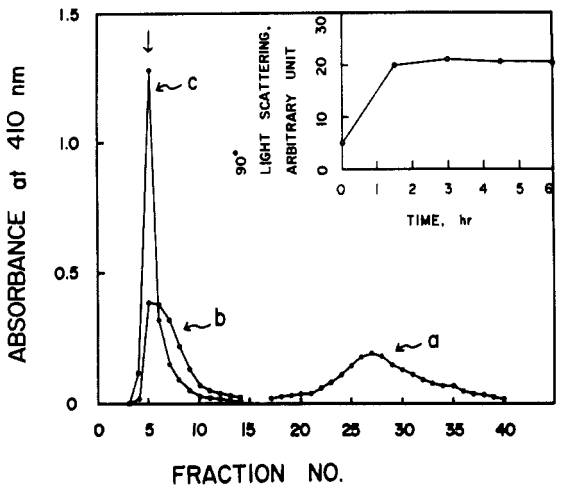

FIGURE 1: Elution pattern of cytochrome *c*/phospholipid–liposome mixtures: (a) obtained by eluting free cytochrome *c* solution; (b) obtained by mixing cytochrome *c* with cardiolipin–lecithin liposomes and eluting at zero time; (c) acquired 3 h after the mixing of cytochrome *c* and cardiolipin–lecithin liposomes. A Sepharose 4B column of 1 × 10 cm was used. It was first presaturated with lecithin liposomes and equilibrated with 10 mM Tris buffer–1 mM EDTA at pH 7.0. The liposome samples were eluted with 10 mM Tris buffer–1 mM EDTA at pH 7.0. Each fraction contained 0.6 mL of eluate. Cytochrome *c* in each fraction was quantitated by the absorbance at 410 nm. Liposomes were prepared as described under Materials and Methods. The arrow (↓) denotes the void volume of the column.

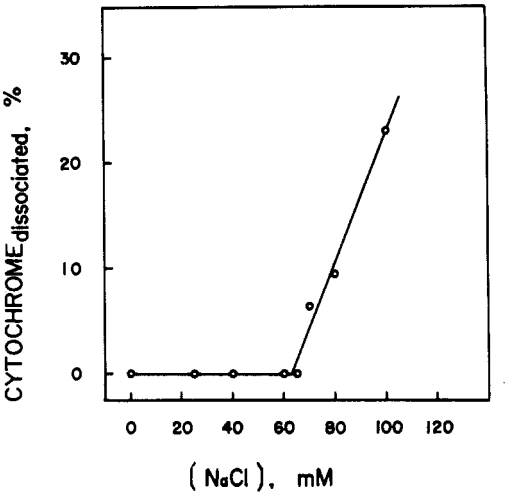

FIGURE 2: Effect of salt concentration on the binding of cytochrome *c* to cardiolipin–lecithin liposomes. Protein–lipid complexes with different salt concentrations were prepared as described under Materials and Methods. After 5 h of incubation at room temperature, sample was applied to a Sepharose 4B column to obtain a chromatogram similar to that in Figure 1. Amount of cytochrome *c* released from liposomes was determined by the absorbance at 410 nm.

cytochrome *c* to phospholipid, phospholipids were isolated from the protein–lipid complexes (Garbus et al., 1963) and chromatographed on a silica gel plate with a solvent system of $CHCl_3$–CH_3OH–CH_3CO_2H–HCO_2H–H_2O (35:15:6:2:1, by volume). Isolated phospholipids were then subjected to perchlorate digestion directly (Hess & Dear, 1975). Phosphate concentrations were determined at 820 nm (Ames & Dubin, 1960). In the study of salt effect, the complex was chromatographed by using a Sepharose 4B column, which was presaturated with lecithin liposomes. A sample of 0.5 mL was applied to the column and eluted with 10 mM Tris buffer of pH 7.0. Absorbance at 410 nm was measured to quantitate the ferricytochrome *c* amount in each fraction.

Spectropotentiostatic Experiments of $E^{0\prime}$ Measurements. The fabrications of an optically transparent thin-layer electrode with a gold minigrid were according to Heineman et al. (1975) with some modifications (Huang & Kimura, 1983). The spectropotentiostatic experiments were performed with a three-electrode system on an Amel Model 551 potentiostat/galvanostat equipped with an Amel Model 566 function generator. The reduction potential ($E^{0\prime}$) of cytochrome *c* was calculated from the Nernst equation:

$$E_{\text{appld}} = E^{0\prime} + \frac{RT}{nF} \ln \frac{[\text{O}]}{[\text{R}]}$$

where [O] and [R] stand for the concentrations of oxidized and reduced forms, respectively. Entropy for the complete cell reaction[2] adjusted to the NHE scale, $\Delta S°$, was determined from the equation (Taniguchi et al., 1980)

$$\Delta S° = (S°_{\text{red}} + S°_{\text{H}^+}) - (S°_{\text{ox}} + \tfrac{1}{2}S°_{\text{H}_2}) =$$

$$(S°_{\text{red}} - S°_{\text{ox}}) - (\tfrac{1}{2}S°_{\text{H}_2} - S°_{\text{H}^+}) = F\left(\frac{dE^{0\prime}}{dT}\right) - 15.6 \text{ eu}$$

Standard free-energy change, $\Delta G°$, for the cell reaction was calculated from the $E^{0\prime}$ value at 25 °C, and the standard enthalpy change, $\Delta H°$, was determined from $\Delta G°$ and $\Delta S°$.

Results

Figure 1 shows the time-dependent changes in size of cytochrome *c* containing liposomes. Curve a was obtained by chromatographing free cytochrome *c* without liposomes. Curve b was acquired at zero time after mixing cytochrome *c* and cardiolipin-containing lecithin liposomes. It shows instant binding between the protein and lipids. The size of liposomes becomes larger with time after mixing (curve c). Light scattering measurements at 90° also showed that after 3 h these liposomes reached a stable size (the data are inserted in Figure 1). The samples similar to that shown in curve c were used in later experiments.

When a mixture of cytochrome *c* and lecithin liposomes without cardiolipin was used, an elution curve similar to curve a in Figure 1 was obtained. We found, however, no binding of cytochrome *c* to the liposomes. It is known that the binding forces between basic cytochrome *c* and acidic cardiolipin are mainly electrostatic (Kimelberg et al., 1970). The binding forces can thus be affected by the salt concentration. Figure 2 shows that when NaCl concentration is above 62 mM, cytochrome *c* starts to dissociate from the membrane-bound state. Therefore, 60 mM NaCl was used to assure the association state of the cytochrome *c* containing liposomes. Since the salt acts as supporting electrolyte in electrochemical titration experiments, this minimum salt concentration is absolutely required.

Free cytochrome *c* without liposomes had a reduction potential of 267 mV at 25 °C in a solution containing 60 mM NaCl, 1 mM EDTA, and 10 mM Tris buffer of pH 7.0. Our value obtained by this spectroelectrochemical method is in good agreement with reported values (Table I). In this study, we used a model system consisting of cytochrome *c*, cardiolipin, and egg lecithin at a molar ratio of 1:14:111[3] to study the effect

[2] Cytochrome $c_{\text{ox}} + {}^1/_2H_2 \rightarrow$ cytochrome $c_{\text{red}} + H^+$.

[3] We used 2.5 mg of cytochrome *c*, 2.2 mg of cardiolipin, and 8.8 mg of egg lecithin to prepare the cytochrome *c*/cardiolipin–lecithin complexes, and 2.5 mg of cytochrome *c* and 8.6 mg of phosphatidylinositol to prepare the cytochrome *c*/phosphatidylinositol complexes. A molecular weight of 1569 was used for cardiolipin on the basis of the linoleoyl form, 785 was used for lecithin on the basis of the oleoyl form, and 885 was used for phosphatidylinositol on the basis of the oleoyl form.

Table I: $E^{0\prime}$ of Horse Heart Cytochrome *c*, Free[a] and Membrane-Bound Forms, As Measured under Various Experimental Conditions

buffer soln compositions[b]	pH	temp (°C)	$E^{0\prime}$	ref
Free				
$I = 0.10$	7.0	25	270	*c*
60 mM NaCl, 10 mM Tris, 1 mM EDTA	7.0	25	267	*d*
60 mM NaCl, 10 mM Tris, 1 mM EDTA, 7 mM PC	7.0	25	267	*d*
0.2 M NaCl, 10 mM Tris, 1 mM EDTA, CL:PC = 1:4 (w/w)	7.0	25	262	*d*
0.1 M NaCl, 0.1 M phosphate	7.0	25	262	*e*
0.1 M NaCl, 0.1 M K-phosphate	7.0	25	262	*d*
0.1 M $NaClO_4$, 0.02 M phosphate	7.0	25	255	*f*
Bound				
60 mM NaCl, 10 mM Tris, 1 mM EDTA, CL:PC = 1:4 (w/w)	7.0	25	251	*d*
0.225 M mannitol, 0.075 M sucrose, 20 mM MOPS, CL:PC = 1:1.33 (w/w)	7.0	22–24	240	*g*
60 mM NaCl, 10 mM Tris, 1 mM EDTA, CL:DPPC = 1:4 (w/w)	7.0	25	233	*d*
0.225 M mannitol, 0.075 M sucrose, 20 mM MOPS, cytochrome *c* depleted mitochondria	7.0	22–24	230	*g*
0.005 M phosphate, 1.25 mg/mL PI (plant)	7.0	25	230	*h*
0.15 M KCl, 0.01 M succinate, CL:PC = 1:4 (w/w)	7.5	22	225	*i*
60 mM NaCl, 10 mM Tris, 1 mM EDTA, 6 mM PI (bovine liver)	7.0	25	217[j]	*d*
60 mM NaCl, 10 mM Tris, 1 mM EDTA, 6 mM PI (soybean)	7.0	25	214[k]	*d*

[a] Only literature values of $E^{0\prime}$ measured by electrochemical method are listed. [b] MOPS, 4-morpholinepropanesulfonic acid; CL, cardiolipin; PC, egg yolk lecithin; DPPC, dipalmitoylphosphatidylcholine; PI, phosphatidylinositol; *I*, ionic strength. [c] Taniguchi et al., 1980. [d] This work. [e] Heineman et al., 1975. [f] Eddowes & Hill, 1979. [g] Vanderkooi et al., 1973b. [h] Erecińska & Vanderkooi, 1975. [i] Kimelberg & Lee, 1970. [j] 104 mV slope. [k] 77 mV slope.

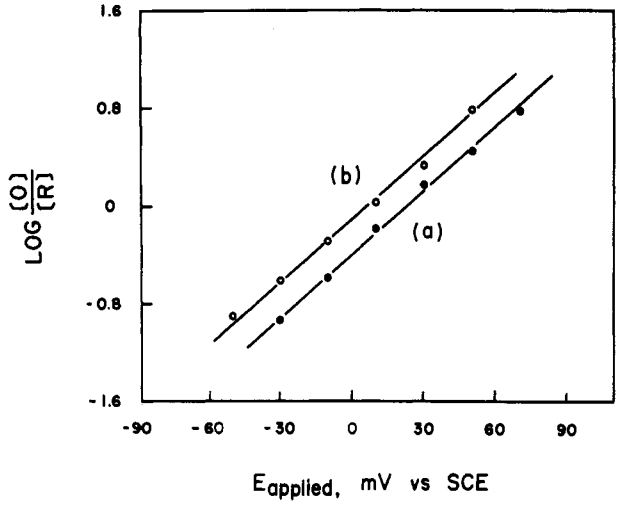

FIGURE 3: Nernst plots of (a) free and (b) membrane-bound cytochrome *c* at 25 °C. The bound form was prepared as in Figure 2. The buffer composition was 10 mM Tris, 1 mM EDTA, and 60 mM NaCl at pH 7.0. Line a gives an $E^{0\prime}$ of 267 mV with a 59 mV slope and a correlation coefficient of 0.998, and line b yields an $E^{0\prime}$ of 251 mV with a 61 mV slope and a correlation coefficient of 0.998.

of membrane. The final cytochrome *c* concentration was approximately 65 μM. The $E^{0\prime}$ value found by our spectropotentiostatic methods was 251 mV at 25 °C and pH 7.0 (Figure 3). Both the free and membrane-bound cytochrome

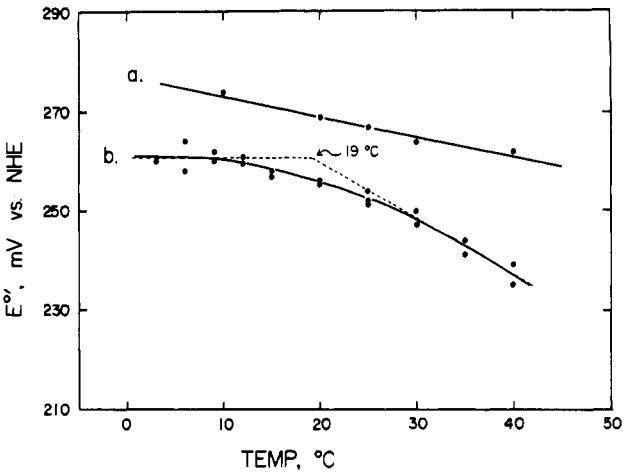

FIGURE 4: Temperature effect on the $E^{0\prime}$ of membrane-free (a) and membrane-bound (b) cytochrome *c*. The bound form was prepared as described under Materials and Methods. Experimental conditions were same as those of Figure 3 except for temperature. Individual titrations gave linear Nernst plots similar to that of Figure 3. Dashed lines in (b) are obtained through the points at 3, 6, 9, and 12 °C and 30, 35, and 40 °C, respectively. The linear least-squares method provided correlation coefficients of 0.984 and 0.941 for line a and line b above 30 °C. The data points below 12 °C had a standard deviation of ±2.2 mV. Alternatively, a line can be drawn through points at 12, 15, 20, and 25 °C. A correlation coefficient of 0.956 was obtained. In this case, the break points are at 11 and 28 °C.

c had very good thermodynamic reversibilities of $n = 1.00$ and 0.98, respectively. Although free cytochrome *c* is insensitive to O_2, membrane-bound cytochrome *c* has been reported as O_2 sensitive (Steinemann & Läuger, 1971; Brown & Wüthrich, 1977). Our results of $E^{0\prime}$ measurements, which have an ideal thermodynamic reversibility of $n = 0.98 \pm 0.03$ ($N = 5$), verify that the interference by O_2 is negligible in our experimental conditions (<90 min). When we used phosphatidylinositol instead of lecithin, a complex of cytochrome *c* with phosphatidylinositol liposomes gave a reduction potential of 214 and 217 mV for soybean and bovine liver phosphatidylinositol, respectively. Our values for phosphatidylinositol liposomes appear to be lower than the reported value of 230 mV by Erecińska & Vanderkooi (1975). The reason for discrepancy is unclear at the present time. When membrane-bound cytochrome *c* was dissociated from liposomes by increasing the salt concentration, the $E^{0\prime}$ value obtained became close to the value of free cytochrome *c*. For instance, when the salt concentration was elevated to 0.2 M, the cytochrome *c*/cardiolipin–lecithin liposome mixture gave a reduction potential of 262 mV, which is close to the value (267 mV) of free cytochrome *c*. In addition, a mixture of cytochrome *c* and lecithin liposomes without cardiolipin gave a reduction potential of 267 mV, which is the same value as free cytochrome *c*.

Cytochrome *c* is the most well-studied hemoprotein. The thermodynamic parameters of horse heart cytochrome *c* have been reported by many investigators (Erecińska & Vanderkooi, 1975; George et al., 1968; Margalit & Schejter, 1970, 1973; Taniguchi et al., 1980; Watt & Sturtevant, 1969). However, only thermodynamic data for the membrane-bound system were reported for phosphatidylinositol liposome-bound cytochrome *c*. Our protein–lipid complex provided another model to study the thermodynamic properties of the oxidation–reduction reaction of cytochrome *c* in the membrane-bound state. Figure 4 shows the temperature effect on the $E^{0\prime}$ values of both membrane-free and -bound cytochrome *c*. The behavior of membrane-bound cytochrome *c* at low temperature is different from that at high temperature, and a possible break point is

Table II: Thermodynamic Parameters of Free and Membrane-Bound Horse Heart Cytochrome *c* at 25 °C

cytochrome *c*[a]	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (eu)	ref
free	−6.13 ± 0.23	−14.2 ± 2.3	−27.0 ± 7.6	*b*
free	−6.15 ± 0.03	−13.5 ± 0.4	−25.0 ± 1.2	*c*
free	−6.1	−9.1 (−13.4)	−10.2 (−24.6)	*d*
c/PI	−5.28	−14.5 ± 0.6	−30.8	*e*
c/CL-PC	−6.02 ± 0.05	−10.5 ± 1.8	−15.2 ± 5.8	*c, f*
c/CL-PC	−5.86 ± 0.17	−18.4 ± 1.6	−42.1 ± 4.8	*c, g*
c/CL-PC	−5.81 ± 0.03	−14.5 ± 0.5	−29.3 ± 1.6	*c, h*

[a] Free, membrane-free cytochrome *c*; *c*/PI, cytochrome *c*/phosphatidylinositol complexes; *c*/CL-PC, cytochrome *c*/cardiolipin-lecithin complexes. [b] Mean of six literature data (Ericińska & Vanderkooi, 1975; George et al., 1968; Margalit & Schejter, 1970, 1973; Taniguchi et al., 1980; Watt & Sturtevant, 1969). [c] This work. [d] Calculated from $E^{0\prime}$ vs. temperature data collected in the temperature range of 25–42 °C with isothermal cell arrangement (Kreishman et al., 1978). Because these data are not corrected for the enthalpy and entropy contribution from the reference electrode half-cell, they are listed separately from the average of six literature values. Parenthetical data are after correction by us. [e] Calculated from $E^{0\prime}$ vs. temperature data collected in the temperature range of 20–40 °C (Ericińska & Vanderkooi, 1975). [f] Calculated from $E^{0\prime}$ vs. temperature data collected in the temperature range of 3–12 °C. (Figure 4b). [g] Calculated from $E^{0\prime}$ vs. temperature data collected in the temperature range of 30–40 °C. (Figure 4b). [h] Calculated from $E^{0\prime}$ vs. temperature data collected in the temperature range of 12–25 °C. (Figure 4b).

at about 19 °C (see the legend of Figure 4). In the absence of membrane, no break point was observed. Table II lists the thermodynamic parameters derived from this figure, in addition to the reported values.

Discussion

On the basis of literature and our own values, Table I lists the reduction potentials of both membrane-free and membran-bound horse heart cytochrome *c*. The $E^{0\prime}$ of the membrane-free type is dependent on the medium pH and buffer concentrations. It ranges between 270 and 255 mV obtained by electrochemical titration. The bound type has a lower $E^{0\prime}$ value (251–214 mV) in general. From our own measurements under identical conditions, the $E^{0\prime}$ of the membrane-bound form had a lower value by 16–53 mV at 25 °C, depending on the nature of the liposomes. The difference in the reported $E^{0\prime}$ decrements of the bound form could be caused by various reasons. (i) By using different kinds of phospholipids, we found that $E^{0\prime}$ was affected to different extents. For example, our study showed that liposomes of cardiolipin–lecithin and cardiolipin–dipalmitoylphosphatidylcholine gave the $E^{0\prime}$ decrements of 16 and 34 mV, respectively. (ii) States of membrane also affect the $E^{0\prime}$. Kimelberg et al. (1970) used cytochrome *c*/cardiolipin–lecithin multilamellar systems and found the $E^{0\prime}$ of cytochrome *c* to be 225 mV, contrary to our $E^{0\prime}$ value of 251 mV. Since the multilamellar or aggregated membrane has an onionlike structure, cytochrome *c* was sandwiched between phospholipid bilayers. It is reasonable to assume that in multilamellar or aggregated membranes protein molecules undergo more severe conformational changes than those loosely attached to liposomal membrane surfaces. Liposomes with different size and curvature may also affect the reduction potential of cytochrome *c*. (iii) It is well known that pH and salt concentration affect the reduction potential of free cytochrome *c* considerably (Table I). This is valid for membrane-bound cytochrome *c* too. (iv) Figure 4 shows the discontinuous profile of temperature effect on the reduction potential of bound cytochrome *c*. Although it is not certain from our observations because of the use of cardiolipin–lecithin liposomes (a broad transition temperature between −15 and −7 °C for egg lecithin), changes in fluidity of the liposomes can be a factor that influences the $E^{0\prime}$ value.

In this study, we found a biphasic nature of temperature-dependent changes in the reduction potential of membrane-bound cytochrome *c*. It is interesting that at high temperatures the bound form showed large changes in both entropy and enthalpy (Table II). Egg yolk lecithin has a melting point between −15 and −7 °C (Szoka & Papahadjopoulos, 1980), and acyl chains of heart cardiolipin are highly unsaturated (98% of the total acyl groups). Since our system contains lecithin, cardiolipin, and protein, the melting point is predicted to be very low. Therefore, temperature in the range 10–30 °C is unlikely to be the transition temperature of the cytochrome *c*/cardiolipin–lecithin system. Kreishman and coworkers reported that in membrane-free chloride solution the temperature dependence of the reduction potentials of horse heart cytochrome *c* was biphasic with a break point of 42 °C (Kreishman et al., 1978; Anderson et al., 1977). Since we did not use temperature higher than 40 °C for cytochrome *c*, we may overlook the corresponding point.

The in situ location of cytochrome *c* is believed to be the outer surface of the inner mitochondrial membrane (Racker et al., 1970). It can be depleted by a medium with high ionic strength and reincorporated by that with low ionic strength (Vanderkooi et al., 1973a,b). At present, little knowledge about the mutual structural perturbation between cytochrome *c* and phospholipid membranes at a wide range of temperature is available in the literature. However, we speculate here as follows: at high temperature (>30 °C) the hydrophobic interaction between cytochrome and phospholipid molecules increases. This hydrophobic interaction, which is a second process following the first ionic interactions in the binding reaction to membrane (Kimelberg & Papahadjopoulos, 1971; Obraztsov et al., 1976), may be important for the penetration of cytochrome *c* into membrane (Papahadjopoulos et al., 1975; Malhotra et al., 1981). As a result, the conformational changes of the protein molecules (Jori et al., 1974) would substantially decrease the reduction potential of the heme site, as suggested by Myer et al. (1979). Consequently, the thermodynamic properties of bound cytochrome *c* at high temperatures are more negative than those of free form (Table II). Ericińska & Vanderkooi (1975) found that in the temperature range of 20–40 °C, the enthalpy and entropy changes of cytochrome *c* reduction in the phosphatidylinositol liposome-bound state to be the same as that of free cytochrome *c*. The different observation can be explained by the structural differences in the phospholipids used. In Figure 4, at the temperature range between 12 and 25 °C, both ΔH° and ΔS° values of the bound form were found to be close to those of the free form. At the temperature range below 12 °C, the thermodynamic parameters of the bound form are more positive than those of the free form. This complexed phenomenon may not be simply explained by the temperature dependence of hydrophobic interactions between the heme protein and membrane. Perhaps, the involvement of changes in structured water molecules must be considered.

The relationship between the binding ability of cytochrome *c* to membrane and the reduction potential can be expressed in the following equation:

$$\frac{K_o}{K_r} = \exp\left(\frac{E^{0\prime}_f - E^{0\prime}_m}{59}\right)$$

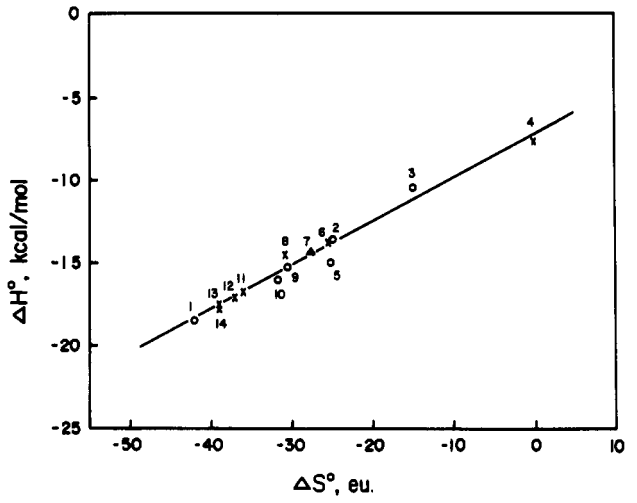

FIGURE 5: $\Delta H°$–$\Delta S°$ diagram for reduction reaction of various cytochrome *c*'s. Data points were calculated from nonisothermal electrochemical titration (O), calorimetry (Δ), and temperature-dependent equilibrium (×). Unless otherwise specified, cytochrome *c* refers to horse heart cytochrome *c*. (1) Cytochrome *c*/cardiolipin–lecithin (>30 °C), this work; (2) cytochrome *c*, membrane-free, this work; (3) cytochrome *c*/cardiolipin–lecithin (<12 °C), this work; (4) cytochrome c_2, Erecińska & Vanderkooi (1975); (5) cytochrome c_2, Taniguchi et al. (1980); (6) cytochrome *c*, average of three literature values, Margalit & Schejter (1970, 1973) and Erecińska & Vanderkooi (1975); (7) cytochrome *c*, average of two literature values, George et al. (1968) and Watt & Sturtevant (1969); (8) cytochrome *c*/phosphatidylinositol, Erecińska & Vanderkooi (1975); (9) cytochrome *c*, Taniguchi et al. (1980); (10) cytochrome c_{551}, Taniguchi et al. (1980); (11) cytochrome *c* from turkey heart, Margalit & Schejter (1970, 1973); (12) cytochrome *c* from bakers' yeast, Margalit & Schejter (1970, 1973); (13) cytochrome *c* from tuna heart, Margalit & Schejter (1970, 1973); (14) cytochrome *c* from *Candida krusei*, Margalit & Schejter (1970, 1973). The linearity with a correlation coefficient of 0.986 gave a slope of 264 K. Krug et al. (1976a,b) reviewed reported enthalpy–entropy compensations in the literature, revealing that errors are associated with determinations of enthalpies and entropies from the slopes of data on van't Hoff plots. The data points (O, Δ) are based on nonisothermal electrochemical titration and calorimetric measurement. The objection is not applicable to these results since van't Hoff plots are not used. However, the literature values (×), which are results of van't Hoff plots, may be subject for consequence of propagation of experimental errors. Nevertheless, regardless of the methods used, all values are fitted well within a straight line. Kreishman et al. (1978) used an isothermal cell for the electrochemical determinations. Their values contain enthalpy and entropy contributions from the reference electrode half-cell. After correction of their values by us, their values were found to be well fitted on the straight line (the data points are not shown in figure). In addition, our $\Delta S°$ and $\Delta H°$ values obtained in the range between 12 and 30 °C (Figure 4b) are also fitted well in the straight line.

where K_o and K_r are the respective association constants of oxidized and reduced cytochrome *c* to membrane. $E^{0\prime}{}_f$ and $E^{0\prime}{}_m$ are the reduction potentials of free and bound cytochrome *c*, respectively. At 25 °C, the reduction potential difference between the free and bound cytochrome *c* is 16 mV, yielding a K_o/K_r of 1.9. At 10 °C, it was calculated to be 1.6. The values for mitochondrial cytochrome *c*, a bound form, were reported to be 3.6–7.0 (Vanderkooi et al., 1973a). Our low values are explained by a difference in phospholipid composition of the membrane used. Yet, a low affinity of reduced cytochrome *c* to membrane was consistently observed in the different membranes.

The large negative value of $\Delta S°$ for the membrane-bound form is presumably due to the increase in compactness of liposomes upon the reduction of cytochrome *c*. It is more difficult to explain the large negative value of $\Delta H°$ at the present time. We can point out, however, some possibilities: (i) when the heme protein is bound to membrane, the π-back-bonding of ferrous ion is enhanced; (ii) if one assumes that the π-back-bonding stabilization of ferrous ion in the bound form is the same as that of the free form ($\Delta H°_f$), the difference between $\Delta H°_b$ of the bound form and ΔH_f of the free form (−18.5 − 14.2) is approximately −4 kcal/mol. The exothermic enthalpy ($\Delta H°_b - \Delta H°_f$) due to the membrane must imply formation of a more rigid membrane structure. This inference is congruent with the observed large negative $\Delta S°$. The enthalpies of main phase transitions of various phosphatidylcholine liposomes are between 5 and 8 kcal/mol (Chen & Sturtevant, 1981). If our assumption is correct, the extent of membrane-structural changes associated with the reduction reaction of cytochrome *c* is less than that of the phase transition.

When $\Delta H°$ values of various cytochrome *c*'s under different conditions were plotted against $\Delta S°$ values, a straight line was obtained with a correlation coefficient of 0.986 (Figure 5). This fact means that all cytochrome *c*'s represent an extrathermodynamic relationship, which is also called the enthalpy–entropy compensation effect. Since these values were collected from various cytochrome *c*'s from different sources and from membrane-bound cytochrome *c*, the relationship stands for a cytochrome *c* family with different amino acid sequences and also for the heme protein in membrane environment. It is safe to say that all cytochrome *c* molecules compensate $\Delta H°$ with $\Delta S°$ in order to maintain the value of $\Delta G°$ as constant as possible. We obtained the compensation factor or isokinetic temperature from the slope as 264 K.

Registry No. Cytochrome *c*, 9007-43-6; dipalmitoylphosphatidylcholine, 2644-64-6.

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Proton-Translocating Adenosinetriphosphatase in Rough and Smooth Microsomes from Rat Liver†

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ABSTRACT: Rat liver smooth and rough microsomal membranes exhibit an ATP-dependent H^+ transport which can be inhibited by sulfhydryl reagents and dicyclohexylcarbodiimide but is resistant to oligomycin. On the basis of inhibitor sensitivities and substrate specificities, this H^+ pump was found to be different from that of mitochondria, lysosomes, gastric H^+–K^+-ATPase, and yeast plasma membrane H^+-ATPase but to resemble that of endocytic vesicles and the H^+ pump responsible for urinary acidification. The transport process is accelerated by valinomycin in the presence of potassium, suggesting that it is an electrogenic pump. The same fractions were enriched in an ATPase with inhibitor sensitivities similar to those of the transport activity. It is possible that the proton electrochemical gradients generated by this pump may play a role in the translocation of proteins and sugars, two of the major functions of these structures.

Intracellular organelles exist frequently as membrane-bound compartments allowing the development of microclimates where the concentration of various substances could be maintained at levels different from those in the cytoplasm. It is now clear that the organellar membranes possess a variety of transport activities that generate gradients of concentration and potential that can be used not only to create these microclimates but also to act as driving forces for the accumulation or extrusion of solutes in these compartments. Recent studies on the function of microsomal vesicles have uncovered the important role that the endoplasmic reticulum plays in the synthesis and packaging of secretory and membrane proteins and in their initial glycosylation. We were stimulated by the observation that monensin, a protonophore which exchanges H^+ for Na^+ or K^+, had dramatic effects on the function and structure of these organelles (Tartakoff & Vassalli, 1978; Ledger et al., 1983). Since one possible mechanism by which monensin could exert its effect is by collapsing a pH gradient in these organelles, we tested microsomes for the ability to transport protons. We found that these membranes contain a proton-translocating ATPase which appears to be different from the mitochondrial F_0–F_1-ATPase and the gastric H^+–K^+-ATPase.

Materials and Methods

Rat liver microsomes were prepared according to the procedure of Adelman et al. (1973). All low-speed centrifugations were performed in a Sorvall Model RC-5B centrifuge and the high-speed spins in a Beckman Model L5-50 ultracentrifuge. All steps were performed at ~4 °C. Briefly, fresh livers were excised, placed in ice-cold 0.25 M sucrose, cut into five pieces each, blotted on filter paper, and forced through a stainless-steel screen with a mesh size of 1 × 1 mm into 1 M sucrose (2 mL/g of tissue). This was homogenized with eight strokes of a motor-driven Teflon pestle and glass homogenizer at 1500 rpm (Caframo type RZR50, Warton, Ontario), filtered

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