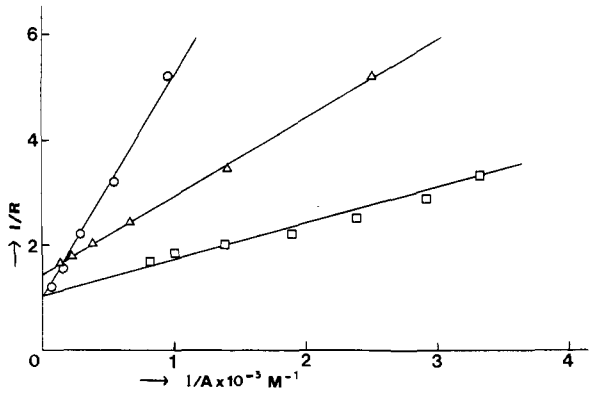

Figure 2. Binding of picrate salts to a crown ether network polymer in dioxane at 25 °C (0.70 mequiv of benzo-18-crown-6 per gram of polystyrene network): (○) sodium picrate; (□) potassium picrate; (△) cesium picrate.

polymer I. It can be obtained by equilibrating the crown resin with a picrate solution in dioxane. The binding in such a system can be described by a rearranged form of the Langmuir adsorption isotherm

$$1/R = 1/n + 1/(nK_NA) \quad (6)$$

where $1/R = Cr_0^*/Pi^-,M^+,Cr^*$, A is the free picrate concentration in solution which can be measured, K_N is the intrinsic binding constant, and $1/n$ denotes the number of crown units in the Pi^-,M^+,Cr^* complex (usually $1/n = 1$ or 2). Plots of $1/R$ vs. $1/A$ for network I in dioxane are depicted in Figure 2. The intercept for Cs^+ deviates from 1, and the value $1/n = 1.40$ suggests the presence of 2:1 crown–Cs^+ complexes on saturation of the network. Such complexes have been found in linear polymers with the benzo-18-crown-6 ligand.[6] The K_N values calculated from the slopes of Figure 2 are 2.58×10^4 (Na^+), 1.72×10^5 (K^+), and 6.55×10^4 M^{-1} (Cs^+, assuming a 1:1 complex).

K_L values for Pi^-,K^+ can now be computed from eq 5 and $K_N = 1.72 \times 10^5$ M^{-1}. They are listed in Table I. A direct spectrophotometric measurement for 4′-(methylbenzo)-18-crown-6 yielded $K_L = 1.7 \times 10^5$ M^{-1}, close to the value found by the network method (the spectral method is rather inaccurate since the shift in λ is only 10 nm). Comparisons with available literature data indicate that the order of K_L values for the four crown ligands of Table I is solvent and anion dependent. Most data in apolar solvents have been derived from extraction experiments which yield composite extraction equilibrium constants. An exception are the interesting ^1H NMR measurements of Reinhoudt et al. in $CDCl_3$[7,8] although this method requires high complex concentrations. A distinct advantage of the method described above is the low ion pair concentration (10^{-4}–10^{-5} M) which minimizes ion pair aggregation.[9] A direct measurement of equilibrium 4 is difficult since Pi^-,M^+ and Pi^-,M^+,L often have nearly identical spectra (Pi^-,M^+,L in dioxane is a tight ion pair for most crown ligands). Cryptand 2.2.2. forms a cryptated loose ion pair (λ_m changes from 349 to 380 nm), but the value of K_L is very high (see Table I) which makes a direct measurement difficult.

The K_N data are important in evaluating the cation binding behavior of crown ether networks as a function of variables such as spacing between crown units (especially important in systems where the cation complexes simultaneously with two crown units), the length and structure of the chain connecting the macrocycle to the polymer, the cross-linking density, presence of comonomer substituents, etc. For ion pairs with anions that have less favorable optical absorption spectra than picrate salts, competitive binding can be employed, i.e., $Pi^-,M^+,Cr^* + A^-,M^+ \rightleftarrows Pi^-,M^+ + A^-,M^+,Cr^*$. The equilibrium constant of this reaction, together with the K_N value for the picrate salt, yields the binding constant K_N for the A^-,M^+ ion pair to the network. Preliminary data for the BPh_4^- anion shows that the binding of this alkali salt to the crown network is much larger than for the corresponding alkali picrates. Similar competitive equilibria in solution can be used to arrive at K_L values in apolar solvents for anions other than picrates.

The above method can also be used to study interactions of ion pairs with additives such as water. For example, small amounts of water added to Pi^-,M^+,Cr^* in dioxane release the picrate and form $Pi^-,M^+,(H_2O)_n$. Networks containing cryptand or podand (e.g., glyme) ligands can also be used in the ion pair studies. Complications will arise in solvents where free ion dissociation becomes significant, since complex formation constants between ligands and free cations are often much higher than between ligands and ion pairs.[10] Some deviations from linearity in the adsorption plots of Figure 2 have been observed when tetrahydrofuran is used as solvent, probably as a result of partial dissociation of the Pi^-,M^+,L ion pairs into free ions.

Acknowledgment. We gratefully acknowledge the financial support of this research by the National Science Foundation (Grant CHE 7905890).

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Efficient Electron Channel through Self-Aggregation of Cytochrome c_3 on an Artificial Membrane

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Received March 16, 1981

Among many known heme proteins, cytochrome c_3 (abbreviated as c_3) is undoubtedly one of the most interesting. It has a unique structure to keep four heme units in a single protein[1] of moderate molecular weight (e.g., ca. 14000 for Desulfovibrio vulgaris Miyazaki[1]) in which each Fe–Fe distance is not too long, ranging between 11.5 and 17.9 Å for D. vulgaris or 10.9–17.3 Å for D. desulfuricans[2,3] and interacting with each other as is apparent from the Mössbauer[4] or EPR[5] spectrum. This electron carrier also has a unique property to conduct electriicity (1.8×10^{-2} Ω^{-1} cm^{-1}) in a solid-state film.[6] This conductivity strongly indicates that this protein has a very strong intermolecular interaction with surrounding proteins.

However, nothing has been clarified about the nature of these significant intramolecular and intermolecular heme–heme interactions, and more mechanistic investigation seems to be necessary and important.

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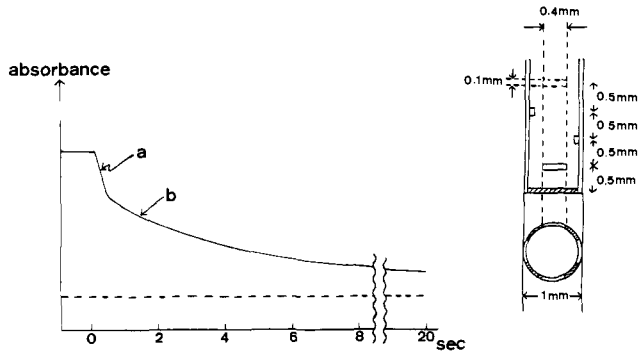

Figure 1. Change in the absorbance at 435 nm. A specially designed syringe is used which has small holes and a closed tip as shown on the right-hand side of Figure 1. After the electron-transport experiments, the artificial liposome was recovered and analyzed by gel filtration and electronic spectroscopy. It is concluded from these experiments that the original shape (effluent volume as well as 215-nm absorption) and composition (398 nmol of $K_3Fe(CN)_6$ remained in the recovered liposome after gel filtration and reoxidation starting from original 400 nmol) were preserved after the electron transport. curve a corresponds to cytochrome c_3 reduction and curve b to ferricyanide reduction.

Some of the authors have studied electron transport across the lecithin membrane which was functionalized with an electron carrier,[7-9] and we are now applying this technique to c_3 mechanism elucidation. We wish to report that the artificial liposomes functionalized with cytochrome c_3 from Desulfovibrio vulgaris Miyazaki efficiently transport electrons across the lecithin–c_3 membrane where a significant intermolecular interaction of c_3 was kinetically demonstrated.

Separation and purification of cytochrome c_3 were carried out according to the literature.[1] An artificial liposome incorporating $K_3Fe(CN)_6$ in its interior was prepared from 80 mg of egg lecithin, carefully purified,[10] and 823 mg of $K_3Fe(CN)_6$ at pH 7.0 (Tris–HCl buffer, 5 mM) with 6 × 5-min ultrasonic irradiation.[11]

The irradiation was carried out in a box filled with Ar to avoid any air oxidation of lecithin, and external cooling was done with ice. The resultant Fe^{III}(i)·Lip (abbreviation of liposome containing Fe^{III} in its interior) was purified through ultracentrifugation and gel filtration on a Sepharose 4B column. Through these procedures, Fe^{III}(i)·Lip was completely separated from free ferricyanide, smaller lecithin aggregates, or multilayer liposome (monitored at 300 nm),[11] and the isolated Fe^{III}(i)·Lip showed reasonable stability where ferricyanide was eluting out in less than 2% during 72 h at 4 °C. Similarly, the liposome functionalized with negatively charged cardiolipin[12] (Fe^{III}(i)·Lip^-) was prepared. To these freshly prepared artificial liposomes (1.0 mL) a desired amount of 60 μM solution of cytochrome c_3 bearing a positive surface charge was added dropwise with gentle stirring for 1 min at room temperature, and the volume of the solution was adjusted to 2.0 mL with the Tris–HCl buffer. The amount of free cytochrome c_3 and that bound to Fe^{III}(i)·Lip^- were analyzed by a Sephadex G-50 column. Totally functionalized liposome, Fe^{III}(i)·Lip^- c_3, thus formed showed the electronic absorptions at 350, 410, and 530 with a shoulder at 560 nm and circular dichroism absorptions at 407 nm (positive).

The liposome solution was mixed with a $Na_2S_2O_4$ solution (pH 7.0, 4-fold excess), and the change of the absorbance at a given wavelength was followed by use of a specially designed stopped-flow apparatus. Immediately after the mixing, rapid convesion of cytochrome c_3^{III} to c_3^{II} was observed which was ascertained by the appearance of absorption at 552 nm until a steady state

Table I. Reduction Rate Constant of $K_3Fe(CN)_6$ in the Functionalized Liposome[a]

cyt c_3, μM	k, s^{-1}	$k/[\text{cyt } c_3]_0^2$, $s^{-1}\ M^{-2}$	cyt c, μM	k, s^{-1}	$k/[\text{cyt } c]_0$, $s^{-1}\ M^{-1}$
8.7	0.149	1.96×10^9	8.7	0.0075	0.86×10^3
6.5	0.080	1.89×10^9	6.5	0.0070	1.08×10^3
4.5	0.038	1.88×10^9	2.9	0.0030	1.03×10^3
3.0	0.016	1.78×10^9			
1.6	0.0028				

[a] $k_0 = 0.0010\ s^{-1}$ (without c_3).

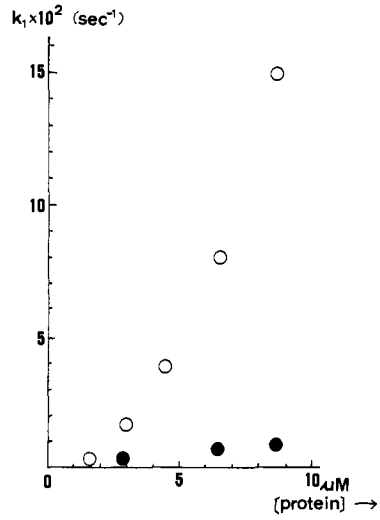

Figure 2. Dependence of k on the concentration of the enzyme. All of the measurements were carried in 5 mM Tris-HCl buffer adjusted to pH 7.0 at 27 °C. (○) cytochrome c_3; (●) cytochrome c.

($c_3^{II}/c_3^{III} \geq 0.93$) was attained within 0.4 s. This rapid process was followed by the slow first-order decrease of ferricyanide (k_1, eq 1) monitored at 435 nm, but this first-order process became gradually[13] slower as shown in Figure 1. Results are summarized

$$-\frac{d[Fe(CN)_6^{3-}]}{dt} = k_1[Fe(CN)_6^{3-}] \quad (1)$$

in Table I and Figure 1. The striking characteristics of Fe^{III}(i) Lip^-·c_3 are (i) very efficient electron transport across the membrane (facilitated electron transport) and (ii) a significant contribution to the second-order kinetics with respect to cytochrome c_3, which is responsible for the efficient electron transport (see Table I and Figure 2).

$$k_1 = k_3[c_3]^2 + k_2[c_3],\ k_3 >> k_2\ (2);\ k_1 = k_{app} - k_0 \quad (2)$$

where k_{app} is the apparent first-order rate constant for ferricyanide reduction in the c_3 liposome, and k_0 is the apparent first-order rate constant for spontaneous ferricyanide reduction in the liposome without c_3.

The second characteristic is very noteworthy, since the average distance, d_{av}, between c_3 molecules on the membrane estimated to be ca. 240 Å by eq 3 seems too large to be expected from any long-range electron transport between c_3 molecules;

$$d_{av} = \left[\frac{(W_l N_0/M_l)(1/N)(4\pi r^2)}{W_c N_0/M_c}\right]^{1/2} \quad (3)$$

where l and c refer to lecithin and c_3, respectively; W, weight of the material used; M, molecular weight; N_0, Avogadro number; N, aggregation number of lecithin to form a liposome.

Instead, two c_3 molecules should associate even for a short period, and through this aggregate an electron is transported much

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(13) The detailed mechanism of this deceleration is now under investigation, but generated potential difference, especially a pH difference across the membrane, seems important according to our preliminary experiments.

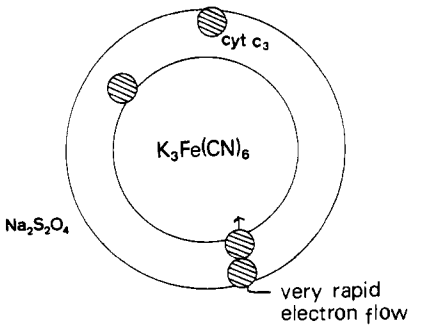

Figure 3. Schematic representation of electron channel in c_3-functionalized liposome. Size of the present c_3 is 33- × 39- × 34-Å,[12] and the thickness of lecithin double layer is 40–50 Å.[15-18]

more efficiently than through isolated c_3. This mechanism is in a good agreement with the observed high electric conductance of the solid c_3 layer, indicating that the intermolecular electron transfer between c_3 is extremely efficient.

Inefficiency of the electron transport through an isolated cytochrome c_3 molecule was compatible with the slow electron transport through the corresponding cytochrome c[14] membrane, $Fe^{III}(i)\cdot Lip^- c$, where an electron was transported only through a first-order kinetic process with cytochrome c (see Figure 2). Therefore, we may draw a conclusion that the "electron channel" formation by the self-aggregation on the artificial membrane is an unique and interesting characteristic of cytochrome c_3 (see Figure 3).[15-18]

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Design and Synthesis of a Model Peptide with β-Endorphin-Like Properties

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We wish to report the synthesis and the characterization of the biological and physical properties of peptide **1** (Figure 1), a model for β-endorphin (Figure 2). On the basis of studies of peptide models of apolipoprotein A-I and melittin, the suggestion has been made that amphiphilic α-helical segments might be important for the biological activities of a variety of peptides which interact with lipid or membrane surfaces.[1] An α-helical structure in the C-terminal region of β-endorphin has previously been postulated to play a role in the receptor binding and opiate activities[2] and resistance to proteolysis[3] of this molecule. We propose here that the β-endorphin molecule consists of the [Met^5]enkephalin region at the N terminus, a hydrophilic "spacer" region from residues 6 through 12, and an amphiphilic helical region between the helix breaker residues[4] Pro^{13} and Gly^{30}. The latter region corresponds either to an amphiphilic α helix with a hydrophobic domain which

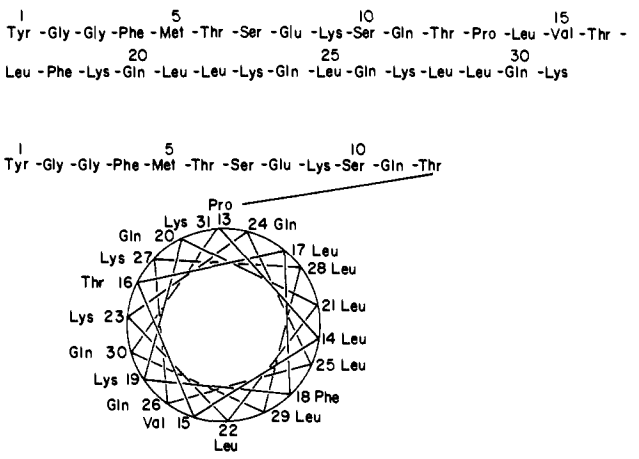

Figure 1. Peptide **1**: amino acid sequence (top) and representation of amphiphilic α-helical segment (bottom) on an Edmundson helical wheel.[12]

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-
Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu

Figure 2. Amino acid sequence of human β-endorphin.

twists around the length of the helix or an amphiphilic π helix,[5] having a hydrophobic domain running straight along the length of the helix.

Because principles for the design of an amphiphilic π helix have not yet been elucidated, we began our development of models for β-endorphin with the synthesis of peptide **1**. As shown in Figure 1, peptide **1** contains a sequence of 31 amino acids having the potential to form an amphiphilic α helix from residues 14 through 31, with a hydrophobic domain running straight along the length of the helix. Peptide **1** has the same amino acid sequence as β-endorphin from residues 1 through 19, which includes the [Met^5]enkephalin region, but has no sequence homology and minimal amino acid residue homology to β-endorphin from residues 20 through 31. In the amphiphilic α or π helix we postulate for β-endorphin residues 14–29, hydrophobic residues cover approximately half of the helix surface, and the hydrophilic residues are either neutral or basic. The peptide **1** sequence from residues 20 through 31 was chosen to reproduce these general characteristics, employing leucines as hydrophobic residues, glutamines as neutral hydrophilic residues, and lysines as basic hydrophilic residues.

Peptide **1** was synthesized by the Merrifield solid-phase method.[6] Cleavage of the peptide from the polymeric support and deprotection was carried out by reaction with anhydrous HF in the presence of anisole at 0 °C. Following extraction of the peptide from its mixture with the resin employing 20% (v/v) aqueous acetic acid and lyophilization, gel filtration was performed on Sephadex G-15 with 0.2 M acetic acid as the eluant. The combined peptide-containing fractions were lyophilized, treated with 10% (w/v) aqueous dithiothreitol solution (0.02 M sodium phosphate buffer, pH 6.6), and then purified by ion exchange chromatography (0.05 M sodium borate buffer, pH 9.0, with a linear gradient of 0–0.25 M NaCl) on CM Sephadex C-25. After lyophilization and desalting, further purification was achieved by partition chromatography on Sephadex G-25 using the solvent system 1-butanol/1-propanol/pyridine/0.2 M aqueous acetic acid (40/19/1/60) followed by lyophilization and gel permeation chromatography (Sephadex G-10, 0.2 M aqueous acetic acid). The yield of pure peptide **1** was 10% on the basis of the crude peptide obtained after the initial Sephadex G-15 gel filtration.

The purified peptide had the expected amino acid composition within experimental error and showed single spots upon TLC in

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