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Binding of a Mitochondrial Presequence to Natural and Artificial Membranes: Role of Surface Potential†

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ABSTRACT: The binding of a synthetic mitochondrial presequence to large, negatively charged, unilamellar vesicles and to unenergized yeast mitochondria has been measured. The presequence, which corresponds to the amino-terminal 25 residues of the yeast cytochrome oxidase subunit IV precursor, was labeled with a fluorescent probe and used to examine the importance of the surface potentials of membranes on the interactions with the presequence. Binding of the fluorescent presequence to the membranes was determined by measuring a decrease in the fluorescence emission of the bound presequence. Binding both to the vesicles and to the mitochondria could be described as a simple partitioning of the presequence between the aqueous and lipid phases. The partitioning was found to depend on the ionic strength of the medium, and the Gouy–Chapman theory could be used to describe the partitioning at various ionic strengths. Application of the theory allowed the determination of an apparent charge on the presequence (+2.31 ± 0.25), salt-independent apparent partition coefficients for vesicles (99 ± 84 M^{-1}) and for unenergized mitochondria (14.5 ± 3.6 L g^{-1}), and an estimated charge density for the mitochondrial outer membrane (−0.0124 ± 0.0016 C m^{-2}). This study shows that electrostatic effects are significant for the binding of a mitochondrial presequence both to lipid vesicles and to mitochondria, the natural target membrane of the presequence. The accumulation of positively charged presequences at the negative mitochondrial surface and the subsequent partitioning of the presequences directly into the mitochondrial outer membrane probably represent early steps in the translocation of precursor proteins into mitochondria.

The targeting of a protein from the cytoplasm to the mitochondria is typically mediated by an amino-terminal targeting sequence, the presequence. Experiments with gene fusions have demonstrated that mitochondrial presequences are necessary and can be sufficient for import of attached proteins into mitochondria (Verner & Schatz, 1988; Hartl et al., 1989). The physical properties of mitochondrial presequences have been studied with synthetic peptides that have sequences corresponding to various natural and artificial presequences (Roise & Schatz, 1988). The synthetic presequences are surface-active, have a strong affinity for membranes containing negatively charged lipids, and adopt secondary structure in hydrophobic environments. It was recently shown that synthetic presequences can be imported into mitochondria in vitro (Ono & Tuboi, 1988; Glaser & Cumsky, 1990; Pak & Weiner, 1990; Furuya et al., 1991; Roise, 1992).

The mechanism of interaction of surface-active peptides with membranes is thought to involve a combination of electrostatic and hydrophobic effects (Sargent & Schwyzer, 1986). Most studies on this process have used model systems, either phospholipid monolayers or bilayers, to mimic the natural membranes (Schoch & Sargent, 1980; Briggs et al., 1985; Roise et al., 1986; Schwarz et al., 1986; Tamm, 1986; Rizzo et al., 1987; Skerjanc et al., 1987; Kuchinka & Seelig, 1989; Seelig & Macdonald, 1989; Beschiaschvili & Seelig, 1990a,b; Frey & Tamm, 1990). While the models allow the experimental conditions to be altered as desired and are amenable to the measurement of binding by a variety of physical tech-

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niques, they do not necessarily reflect the exact behavior of the biological membranes. In order to analyze the binding to natural membranes, other approaches must be taken.

A recent study has shown that a fluorescently labeled, synthetic presequence can be used to monitor the import of the presequence into isolated yeast mitochondria (Roise, 1992). A similar approach has been used here to measure the binding of the presequence both to phospholipid vesicles and to isolated mitochondria. By determining the effect of ionic strength on the affinity of the presequence for membranes, the contribution of the membrane surface potential to the binding could be determined. The results demonstrate that the binding of a presequence to membranes depends strongly on the surface potential of the membrane and that the mechanism of interaction is probably similar for both vesicles and mitochondria. A physical understanding of the early, nonspecific interactions between presequences and mitochondria should facilitate the elucidation of the molecular basis for the specific uptake of precursors by the organelle.

Materials and Methods

Peptide Synthesis and Labeling. A peptide corresponding to the first 25 amino acid residues of the wild-type yeast precursor of cytochrome *c* oxidase subunit IV (MLSLRQSIRFFKPATRTLCSSRYLL-NH_2) was synthesized and characterized as described (Roise et al., 1986). The presequence was labeled with 5-(iodoacetamido)-fluorescein and purified as described (Roise, 1992). The concentration of the presequence was determined by amino acid analysis.

Absorbance Spectra of the Fluorescent Presequence. Absorbance was measured on a Hewlett-Packard 8452 diode array UV–visible spectrophotometer. Samples were at room temperature in a buffered solution containing 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)[1]–KOH, pH 7.2, 10 mM KCl, 0.58 M D-sorbitol, and 0.5 mg mL^{-1} fatty acid free bovine serum albumin (FAF-BSA). Trypsin was added from a 10 mg mL^{-1} stock in the same buffered solution.

Preparation of Extruded Large Unilamellar Vesicles (ELUV). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (25 mg) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (100 mg) (Avanti Polar Lipids, Birmingham, AL) were dissolved in $CHCl_3$. Cholesterol (16 mg) was added, and the solution was mixed. The $CHCl_3$ was removed by rotary evaporation, and the residue was hydrated in a solution containing 10 mM HEPES–KOH, pH 7.2, and 0.5 mg mL^{-1} FAF-BSA (standard buffer). The solution also contained various concentrations of KCl as noted and D-sorbitol to give a final, constant osmotic strength of 0.6 osM.[2] The lipid solution was frozen in liquid nitrogen and thawed at 30 °C 5 times. The solution was passed 10 times through a 100-nm polycarbonate filter in a liposome extruder (Lipex Biomembranes Inc., Vancouver, British Columbia). (Hope et al., 1985).

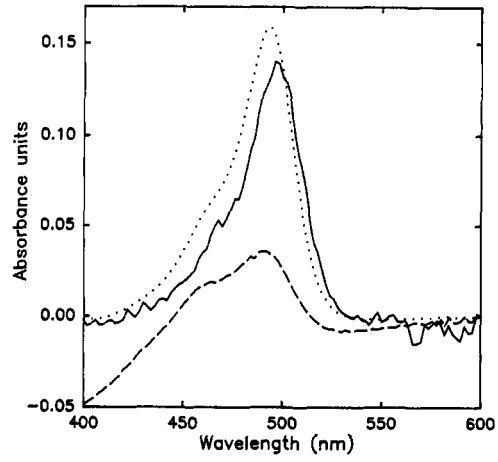

FIGURE 1: UV–visible absorbance spectra of the fluorescent presequence in the presence and absence of ELUV. Absorbance of the presequence alone (2.1 μM, solid line). Difference spectrum calculated by subtracting the spectrum of ELUV alone from the spectrum of the presequence (2.1 μM) in the presence of POPC/POPG/cholesterol (4:1:1.25) ELUV (8.3 mM, dashed line). Difference spectrum calculated by subtracting the spectrum of ELUV and trypsin alone from the spectrum of the presequence (2.1 μM) in the presence of ELUV (8.3 mM) and trypsin (60 μg mL^{-1}) (dotted line).

Isolation of Mitochondria. Mitochondria were purified from yeast strain D273-10B (Daum et al., 1982; Kozlowski & Zagorski, 1988). Protein concentrations of mitochondria were estimated by measuring the absorbance at 280 nm of mitochondria dissolved in 1% SDS(ϵ_{280} = 2.1 cm^2 mg^{-1}).

Binding Experiments. All binding assays were performed at 20 °C. For binding of the fluorescent presequence to ELUV, the solutions used were identical with those used to extrude the ELUV (standard buffer + KCl + D-sorbitol). For binding of the presequence to mitochondria, the solutions also contained 1 mM 1,10-phenanthroline to inhibit a mitochondrial peptidase activity (Roise, 1992). ELUV or isolated mitochondria were diluted into a buffered solution (3-mL total volume) in a quartz cuvette (1 cm × 1 cm). The fluorescent presequence was added stepwise from a concentrated stock solution (50% ethanol) and stirred briefly with the needle of a syringe. The total volume of fluorescent presequence added was always less than or equal to 10 μL. Fluorescence was monitored with an SLM/Aminco SPF-500C fluorometer (excitation 490 nm, emission 518 nm, 5-nm band-pass).

Results

The fluorescent presequence has been shown to bind reversibly to the surface of mitochondria (Roise, 1992). In those experiments, binding was assessed either by measuring cosedimentation of the fluorescence with mitochondria or by observing a decrease in the fluorescence of the presequence that occurred upon binding. Binding of the presequence to negatively charged phospholipid vesicles also causes a decrease in the fluorescence of the labeled presequence. This decrease results from the decreased absorbance of the presequence in the bound form (Figure 1). The change in absorbance is reversible, since treatment of solution containing the bound presequence with trypsin restores the original level of absorbance of the unbound form (Figure 1). Ultracentrifugation of mixtures of the presequence and ELUV showed that the intact presequence was almost completely associated with the vesicles under these conditions while the presequence digested by trypsin showed no association with the ELUV (data not

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FAF-BSA, essentially fatty acid free bovine serum albumin; ELUV, extruded large unilamellar vesicle(s); POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; EDTA, (ethylenedinitrilo)tetraacetic acid; SUV, small unilamellar vesicle(s); NBD, 7-nitro-2,1,3-benzoxadiazole.

[2] BSA and sorbitol were needed to stabilize the isolated mitochondria and were included in the vesicle solutions to maintain identical experimental conditions. Control experiments have shown that these components do not affect the interactions of the presequence with membranes. Other control experiments demonstrated that the addition of low concentrations of EDTA did not affect the observed binding of the presequence to membranes. Effects of any contaminating divalent metals on the surface potentials are, therefore, likely to be negligible.

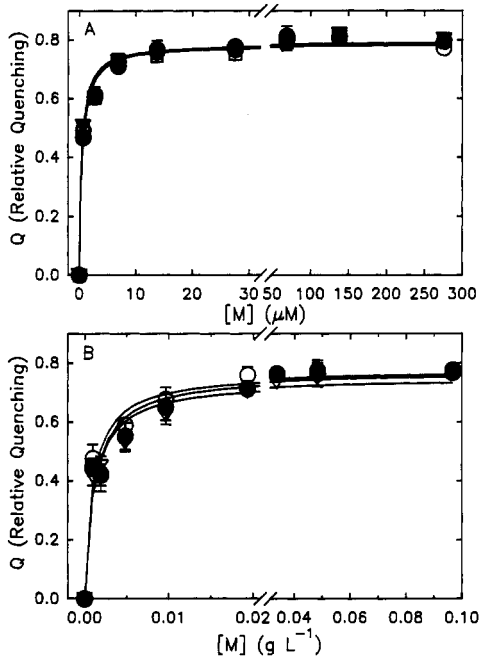

FIGURE 2: Binding of the labeled presequence to membranes in buffered solution containing 10 mM HEPES–KOH, pH 7.2 10 mM KCl, 0.58 M sorbitol, and 0.5 mg mL^{-1} BSA. Concentration of presequence: (○) 10 nM; (●) 20 nM; (▽) 30 nM; (▼) 40 nM; (□) 50 nM. The points were fit to eq 1, and the resulting values of M_{50} were used to calculate K_{app}' values according to eq 2 (Table I). The error bars indicate ±standard deviation; where error bars are not visible, the symbols are larger than the error bars. (Panel A) Binding of the presequence to ELUV. [M] is the total concentration of lipid. (Panel B) Binding of the presequence to isolated mitochondria. [M] is the total concentration of mitochondrial protein.

shown). The decreased absorbance of the bound presequence is probably due to partial protonation of the fluorescein ($pK_a \approx 5.5$) on the surface of the bilayer, since the pH directly adjacent to a negatively charged membrane may be as much as 1–2 pH units lower than the bulk pH due to electrostatic effects (McLaughlin, 1977). The decrease in absorbance of the bound presequence results in a comparable decrease in the fluorescence emission of the presequence. This change in the fluorescence can be used as an extremely sensitive assay to follow binding of the presequence to ELUV and to isolated mitochondria.

The fluorescence of the presequence was measured as a function of membrane concentration for both phospholipid ELUV and isolated yeast mitochondria. The results of experiments where the concentration of KCl was 10 mM are typical (Figure 2). Relative quenching (Q) is defined as $(F_{total} - F)F_{total}^{-1}$, where F_{total} is the fluorescence of the presequence without membranes and F is the fluorescence observed in the presence of membranes. The binding data are consistent with a two-state equilibrium between the membrane-bound, quenched form of the presequence and the free, unquenched form of the presequence. Binding curves were analyzed as previously described (Roise, 1992). The points were fit to an equation describing a rectangular hyperbola:

$$Q = Q_{max}[M](M_{50} + [M])^{-1} \quad (1)$$

In this equation, Q_{max} is a constant that represents the quenching of the bound presequence, [M] is the concentration of the membrane, and M_{50} is a constant that describes the concentration of membrane required for half-maximal quenching. When [M] = M_{50}, half of the total concentration of the presequence is bound, and $[P_B] = [P_F] = 0.5[P_T]$. In this notation, $[P_B]$ and $[P_F]$ represent the bulk concentrations of bound and free presequences, respectively, and $[P_T]$ is the total bulk concentration of the presequence. In the analysis of the binding of peptides to membranes, the concentration of the bound form of the peptide is generally expressed with respect to the total concentration of membrane, so that $r = [P_B][M]^{-1}$ (Schwarz et al., 1986). From the considerations above, it follows that $r = 0.5[P_T]M_{50}^{-1}$ and $[P_F] = 0.5[P_T]$ when [M] = M_{50}.

Table I: Apparent Partition Coefficients (K_{app}') at Various Concentrations of Salt[a]

[salt] (mM)[b]	K_{app}' for ELUV (M^{-1})	K_{app}' for mitochondria (L g^{-1})
3.3	$(4.3 \pm 0.8) \times 10^6$	$(6.1 \pm 1.0) \times 10^3$
13.3	$(2.0 \pm 0.2) \times 10^6$	$(7.0 \pm 0.1) \times 10^2$
28.3	$(8.1 \pm 4.0) \times 10^4$	$(5.0 \pm 2.0) \times 10^2$
53.3	$(1.4 \pm 0.5) \times 10^4$	$(8.9 \pm 2.0) \times 10^1$
103.3	N/D[c]	$(5.1 \pm 0.7) \times 10^1$
203.3	$(2.2 \pm 0.2) \times 10^3$	N/D

[a] K_{app}' values are the result of four groups of measurements done in two separate experiments for each concentration of salt. Values of r and $[P_F]$ were fit to eq 2. The results are presented as mean ± standard deviation. [b] The concentrations of salt include the fraction of the buffer that is charged at pH 7.2. [c] Not determined.

Values of M_{50} at a given salt concentration were found to be independent of the concentration of the presequence for both ELUV and mitochondria. This observation demonstrates that there is a linear relationship between the concentration of the presequence in the membrane, r, and the concentration of the presequence in the aqueous phase, $[P_F]$. The results are consistent with recent reports that the binding can be treated as a simple partitioning of the presequence between the aqueous phase and the membrane phase (Frey & Tamm, 1990; Roise, 1992). The binding remained linear even at the highest values of r. This shows that the binding sites do not saturate under these conditions, either for ELUV or for mitochondria. A linear binding relationship also suggests that there are no significant attractive or repulsive interactions between presequences within the membranes or in the aqueous phase at these concentrations (Schwarz et al., 1986).

A series of binding experiments with ELUV and isolated mitochondria was performed at different concentrations of salt to determine the effects of electrostatic interactions on the binding process. For each concentration of salt, values of M_{50} were determined at various concentrations of the presequence as in Figure 2. The values of M_{50}, which were constant at each concentration of salt, could be used to generate a series of apparent partition coefficients, K_{app}', where

$$K_{app}' = r[P_F]^{-1} = M_{50}^{-1} \quad (2)$$

The units of K_{app}' are liters per mole of lipid for vesicles and liters per gram of mitochondrial protein for mitochondria. The values of K_{app}' derived from experiments with ELUV and mitochondria at various concentrations of salt are compiled in Table I. It is clear that the concentration of salt has a large influence on the partitioning of the presequence into both ELUV and mitochondrial membranes and that the relative size of the effect is similar in each case. Effects of the concentration of salt result from the shielding of attractive electrostatic interactions between the positively charged presequence and the negatively charged membrane; higher concentrations of salt cause a reduction in the observed binding of the presequence to the membranes.

The Gouy–Chapman theory of diffuse double layers has been used to explain the effects of surface potentials on the partitioning of charged peptides into lipid bilayers (Sargent

et al., 1988, 1989; Schwarz & Beschiaschvili, 1989; Seelig & Macdonald, 1989; Beschiaschvili & Seelig, 1990a,b; Frey & Tamm, 1990). The theory explains the effect of a charged surface on ions in the bulk solution. In this case, the concentration of the presequence adjacent to the surface of the membrane, $[P_M]$, is related to the concentration of the presequence in bulk solution, $[P_F]$, by a Boltzmann function (McLaughlin, 1977):

$$[P_M] = [P_F] \exp[-z_p\psi_0 F_0(RT)^{-1}] \quad (3)$$

In this equation, z_p is the charge on the presequence, ψ_0 is the potential at the surface of the membrane, F_0 is the Faraday constant, R is the universal gas constant, and T is the absolute temperature. The surface potential is dependent on the charge density of the membrane, σ, and the molar concentration of the electrolyte in solution, C_e, according to

$$\sigma = [8(1000 \text{ L m}^{-3})C_e\epsilon_r\epsilon_0 RT]^{1/2} \sinh [z_e\psi_0 F_0(2RT)^{-1}] \quad (4)$$

Note that this equation is valid only for symmetric electrolytes (McLaughlin, 1977) and that the surface potential is dependent on the dielectric constant of the solution, ϵ_r, the permittivity of a vacuum, ϵ_0, the charge of the electrolyte, z_e, and the temperature. Note also that the equation ignores the effects of the adsorption of ions from the electrolyte onto the surface of the membrane (Eisenberg et al., 1979).

Equation 4 can be used to calculate values of ψ_0 if the charge density of the membrane is known. The charge density of the ELUV can be calculated directly from the known composition of the lipid mixture and the known surface area per lipid molecule. The molecular areas of POPC (70 Å^2), POPG (70 Å^2), and cholesterol (37 Å^2) were estimated from monolayer studies that had used the same lipid composition as the ELUV used in our experiments (Evans et al., 1987). From the surface areas of the individual lipids, the molar composition of the ELUV, the assumption that only half of the lipids are present in the outer leaflet of the ELUV, and the known fraction of POPG in the vesicles, a charge density of the vesicles was obtained (σ = –0.04043 C m^{-2}). Because of the relatively low values of r observed under the conditions of the binding experiments, the surface charge was not changed significantly by the binding of the cationic presequence, and no corrections have been made for the surface charge of membranes with bound presequences. Likewise, the charge density was estimated to be decreased only slightly by the specific adsorption of potassium ions from the bulk solution (Eisenberg et al., 1979), and the σ value was not corrected for this small effect.

The apparent partition coefficients shown in Table I (K_{app}') vary with the concentration of salt. Apparent partition coefficients that are independent of the salt concentration can be defined for both vesicles and mitochondria by

$$K_{app} = r[P_M]^{-1} = [P_B]([M][P_M])^{-1} \quad (5)$$

This relationship is similar to eq 2, except that the concentration of the presequence adjacent to the membrane, $[P_M]$, is used in place of the bulk concentration of free presequence, $[P_F]$. Substitution of eq 3 into eq 5 and rearrangement give

$$K_{app}' = K_{app} \exp[-z_p\psi_0 F_0(RT)^{-1}] \quad (6)$$

Equations 4 and 6 can be combined and simplified using the identity $\sinh^{-1} X = \ln [X + (X^2 + 1)^{1/2}]$ to give

$$K_{app}' = K_{app}\{[\sigma + (\sigma^2 + C_eB)^{1/2}](C_eB)^{-1/2}\}^{-2z_p/z_e} \quad (7)$$

where $B = 8(1000 \text{ L m}^{-3})\epsilon_0\epsilon_r RT$.

A linear form of eq 7 was used to fit the binding data from the vesicle experiments (Table I). The best fit gave values of $K_{app} = 99 \pm 84$ M^{-1} and $z_p = +2.31 \pm 0.25$. The value for z_p is smaller than expected from the number of ionizable groups in the presequence and represents an effective charge for the presequence. This smaller charge value is probably due to the large size of the presequence relative to the distance from the surface at which the full surface potential is felt. Similar results have been obtained in other systems (Tamm, 1986; Seelig & Macdonald, 1989; Beschiaschvili & Seelig, 1990a,b; Stankowski & Schwarz, 1990; Frey & Tamm, 1990), and several theoretical treatments of the underlying reasons for the observed effect have been reported (Carnie & McLaughlin, 1983; Langner et al., 1990; Stankowski, 1991). In the experiments presented here, the variable concentration of salt may also have an influence on the calculated effective charge of the presequence. The Debye length, which is an indication of how rapidly the electrical potential decays as a function of the distance from a point charge or a charged surface, becomes smaller as the concentration of salt increases (McLaughlin, 1977). This implies that a charged molecule of finite size is more likely to appear fully charged at low concentrations of salt where the Debye length is larger relative to the size of the molecule. Indeed, a recent study found that a polycationic peptide appeared fully charged in 10 mM KCl but had an effective charge of half as much in 100 mM KCl (Mosior & McLaughlin, 1992). A similar effect had previously been reported for the binding of melittin to neutral vesicles (Stankowski & Schwarz, 1990). Thus, the value of z_p estimated from the presequence binding data is actually an average of effective charges over the different concentrations of salt.

A plot of K_{app}' as a function of the concentration of salt shows good agreement between the experimental data for the binding of the presequence to ELUV (Table I) and a line generated with eq 7 using $K_{app} = 99$ M^{-1} and $z_p = +2.31$ (Figure 3A). A similar approach was used to analyze the binding data for mitochondria. For these purposes, it was assumed that the effective charge of the presequence, z_p, is the same for the experiments with vesicles and for those with mitochondria. This value (+2.31) was used in a linear form of eq 7 to fit the experimental points for the binding of the presequence to mitochondria (Table I). Results of the fit gave values of $\sigma = -0.0124 \pm 0.0016$ C m^{-2} and $K_{app} = 14.5 \pm 3.6$ L g^{-1}. A line generated by eq 7 using these values over the range of C_e shows a good agreement with the data points (Figure 3B).

Discussion

The mechanism of interaction of charged, surface-active peptides with model membranes has been the subject of many recent studies (Kuchinka & Seelig, 1989; Sargent et al., 1989; Schwarz & Beschiaschvili, 1989; Seelig & Macdonald, 1989; Beschiaschvili & Seelig, 1990a,b, 1991; Frey & Tamm, 1990). In each case, the binding could be explained by a relatively simple combination of events. First, the charged peptide is accumulated or depleted at the surface of a charged membrane due to the effects of the diffuse double layer. These interactions are described by the Gouy–Chapman theory. Second, the peptide in the local pool adjacent to the membrane partitions directly into the phospholipid bilayer. The partitioning depends on nonspecific contacts between the peptide and the hydrophobic core of the bilayer as well as any specific interactions between the bound peptide and components of the bilayer.

In the current study, a similar approach was applied to the interactions between a mitochondrial presequence and two target membranes: large unilamellar vesicles and purified

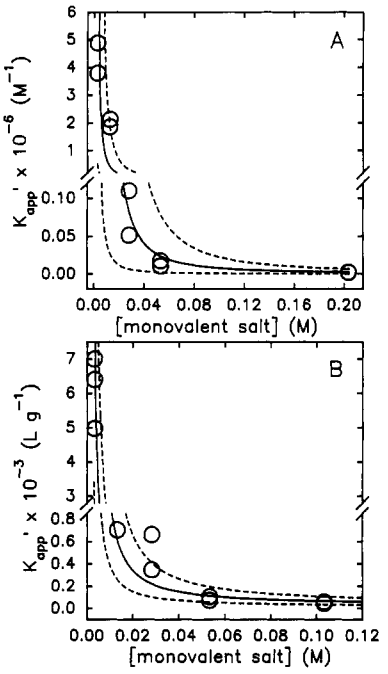

FIGURE 3: Effects of the concentration of salt on the binding of the presequence to membranes. (Panel A) K_{app}' values from binding experiments with ELUV are plotted against the concentration of salt. The solid line was generated using eq 7 with $K_{app} = 99\ M^{-1}$, $z_p = 2.31$, and $\sigma = -0.04043\ C\ m^{-2}$. (Panel B) K_{app} values from binding experiments with mitochondria are plotted against the concentration of salt. The solid line was generated using eq 7 with the values of $K_{app} = 14.5\ L\ g^{-1}$, $z_p = +2.31$, and $\sigma = -0.0124\ C\ m^{-2}$. The dashed lines in each panel represent curves generated from eq 7 using extreme values (mean ± standard error) from the fitting procedure.

yeast mitochondria. It has recently been shown that the binding of the surface-active presequence to mitochondria involves insertion of the presequence into the lipid phase of the mitochondrial outer membrane and that translocation of the presequence across the mitochondrial membranes probably occurs from this membrane-bound pool (Roise, 1992). Those experiments made use of a fluorescent, synthetic presequence that allowed the interactions between the presequence and mitochondria to be monitored spectroscopically at physiologically relevant concentrations of the presequence and with minimal interference of the signal by the biological membranes. The same system has now been applied specifically to the initial binding step of the presequence to mitochondria in order to distinguish the relative importance of various interactions in the binding process.

The effects of the negatively charged membranes on the cationic presequence have been modulated by varying the salt concentration of the medium in the binding assays. In order to apply the Gouy–Chapman theory to this system, the effective charge on the presequence had to be determined. This was done by measuring the effect of salt on the binding of the presequence to membranes of known charge density. The resulting apparent partition coefficients at each concentration of salt could be fit to eq 7. The fit yielded an effective charge on the presequence, $z_p = +2.31 \pm 0.25$, and a potential-independent, apparent partition coefficient, $K_{app} = 99 \pm 84\ M^{-1}$. These results can be compared to a recent report of Frey and Tamm (1990). In that work, the same presequence was labeled with 7-nitro-2,1,3-benzoxadiazole (NBD), and the binding to small unilamellar vesicles (SUV) that contained mixtures of POPC and POPG was monitored by enhancement of fluorescence. Using Gouy–Chapman analysis, they estimated $K_{app} = (3.9 \pm 0.8) \times 10^3\ M^{-1}$ and $z_p = 1.8 \pm 0.1$. The differences in K_{app} values obtained in the two studies may be due to the type of model membrane used in each case. Unlike natural membranes, SUV have a highly curved surface, and the packing of lipid molecules is asymmetric (Huang & Mason, 1978). The ELUV used here are significantly less curved than SUV. We have observed that binding of the presequence to SUV resulted in a much larger K_{app}' than that observed for ELUV in the same buffer (unpublished data) and similar effects have been reported for other systems as well (Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990a). It will be important to determine how general the effect of size on the binding properties of vesicles is, since SUV are so commonly used as models for biological membranes. An additional difference between the two systems was the presence of cholesterol in the vesicles used here. It is not clear what effect the presence of cholesterol would have on the interactions between the presequence and model membranes, although cholesterol was found to decrease the affinity of alamethicin for neutral membranes by 2–3-fold (Stankowski & Schwarz, 1989).

The values of z_p estimated for the presequence in the two systems were similar, although both were much lower than expected from the actual charge on the presequence (+4 to +6 depending on the label). This observation is not surprising, since the Gouy–Chapman theory assumes that the ions in solution are point charges. This assumption does not hold for large molecules, and the concentrative effect observed is often less than would be predicted by classical theory (Alvarez et al., 1983). At high concentrations of salt, where the Debye length is small relative to the size of the charged molecule, the discreteness of charge effect can become significant, and the effective charge of the peptide decreases. The effect of the salt concentration on the effective charge may also explain why the z_p reported by Frey and Tamm (1990) is somewhat smaller than that determined here, since the concentration of salt used in that work was 150 mM, while that used here was in a range that was mainly below this concentration. Similar effects have been reported by Mosior and McLaughlin (1992).

Perhaps the most important aspect of this work is the analysis of presequence binding to isolated mitochondria. The effect of salt on the binding was found to be qualitatively similar to that observed with ELUV (Table I). The apparent partition coefficients at each concentration of salt were used, together with the z_p obtained from the vesicle experiments, to calculate values of σ and K_{app} for the mitochondria. A line generated using these values according to Gouy–Chapman theory shows excellent agreement with the experimental points (Figure 3B) and demonstrates that the contribution of the diffuse double layer to interactions between a presequence and the surface of mitochondria can be significant. It is assumed that the presequence does not diffuse significantly through the pores of the mitochondrial outer membrane during the time course of the binding assays, so the charge density obtained from the binding measurements reflects lipids and proteins associated with the outer leaflet of the outer membrane.

The σ for mitochondrial membranes measured in this work corresponds to a charge of −1 per 1300 Å^2 of membrane surface area. Surprisingly, this charge density is roughly 3-fold lower than expected from the known lipid composition of the outer mitochondrial membrane (Daum, 1985). This suggests that much of the negative lipid in the membrane may not be available to interact with the presequence, perhaps because it is neutralized by positively charged proteins embedded in the membrane. The commonly used value of 10–20% for the fraction of negative lipids in natural membranes (McLaughlin,

1977; Daum, 1985) may, therefore, be an overestimate of the effective charge density of the mitochondrial outer membrane.

Work presented here suggests that electrostatic interactions of mitochondrial presequences with the outer membrane of the mitochondria may be involved in the import of precursor proteins into mitochondria. In a eukaryotic cell, it is likely that other membranes would have surface potentials similar to that of the mitochondria and that a reversible, relatively weak, nonspecific association of precursors with all negatively charged membranes may occur. The ultimate specificity of the import of precursors into mitochondria may be the result of specific interactions of the bound presequence with receptor components within the mitochondrial outer membrane and/or the irreversible, $\Delta\psi$-dependent translocation of the bound presequences across the mitochondrial membranes (Roise, 1992). Although the overall mechanism of protein translocation into mitochondria remains unknown, the initial association of the presequence with the surface of the mitochondria may be understood by the fundamental physical principles described here.

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