Time Resolution of Binding and Membrane Insertion of a Mitochondrial Signal Peptide: Correlation with Structural Changes and Evidence for Cooperativity†

Caroline Golding,‡ Simon Senior,§ Michael T. Wilson,‡ and Paul O'Shea*,‡

Department of Biological & Chemical Sciences, University of Essex, Colchester, CO4 3SQ Essex, U.K., and Applied Photophysics Ltd., Leatherhead, KT22 7PB Surrey, U.K.

Received April 15, 1996; Revised Manuscript Received June 10, 1996⊗

ABSTRACT: Utilizing a recently developed novel fluorescence technique [Wall et al. (1995) *Mol. Membr. Biol. 12,* 183−192], it is shown that the interactions of p25, the leader peptide of subunit IV of cytochrome *c* oxidase, with phospholipid membranes can be identified in real time. p25 is observed to bind following stopped-flow mixing of the peptide with phospholipid membranes with rate constants up to about 700 s^{-1} and then insert into the membrane with rate constants on the order of 0.4 s^{-1}. Comparison of these processes with similarly time-resolved experiments performed with a stopped-flow CD spectrometer revealed that p25 does not become α-helical upon binding to the membrane. Following membrane insertion, however, p25 was observed to adopt an α-helical configuration. The temperature dependency of these processes was then found to yield activation energies for the respective components of the p25−membrane interaction.

A number of factors appear to be necessary to facilitate the targeting and insertion of newly synthesized proteins into membranes. These include attachment to the incipient membrane protein of a signal/leader sequence oligopeptide (LSO) which aids the membrane insertion process and specifies the target membrane. Complementing these primary sequence modifications of the nascent protein, transport systems in the target membrane also appear to be required to promote the membrane insertion/translocation events [e.g., Blobel and Dobberstein (1975)]. It has also been suggested that interactions between different membranes may also be a feature of some intracellular targets. Both the inner (MIM) and outer (MOM) mitochondrial membranes (Keibler et al. 1993; Stuart et al., 1994; Mayer et al., 1995), for example, are thought to be involved in at least some of the protein transport mechanisms in this organelle. The explicit mechanism which underlies the molecular recognition process between the LSO and the putative receptor is thought to involve direct protein-protein interactions either on or within the membrane (Gilmore & Blobel, 1985).

Many studies have been directed toward understanding the nature of the interactions of leader peptides with a simple phospholipid membrane [e.g. reviewed by Tamm (1991)]. While these studies have clearly shown that virtually all types of LSO possess great affinities for such membranes (Maduke & Roise, 1993; Leenhouts et al., 1995), *in vivo*, the target is most likely a protein complex located within the corresponding membrane which facilitates the translocation, presumably by a channel type of mechanism (Keibler et al., 1993). Recently, it has been shown that such membrane-located peptide channels may also be openly accessible to the hydrophobic domains of the lipid membrane (Martoglio et al., 1995). In other words, given the large affinity of the leader/signal peptides for the lipid phase of the membrane, it is a strong possibility that their target "binding sites" may be within the lipid core of the membrane. On the other hand, some of these proteins also project from the membrane surface and possess negative charges (e.g. MOM22/TOM22) and have been suggested to be involved in the binding of the peptide in the more polar environment of the membrane−solution interface (Keibler et al., 1993; Mayer et al., 1995).

These latter possibilities should not be seen to be mutually exclusive; rather, they may simply reflect different aspects of the complex nature of the recognition mechanism between the target membrane and the leader/signal peptide. Perhaps, in a related manner, it has long been a point of concern that leader peptides do not seem to possess unique primary sequence homologies which correspond to particular membrane targets. An interesting question to be addressed, therefore, is given the similarity of this peptide to other such leader sequences, where does the targeting information lie? Presently, although LSOs certainly are able to specify a particular target membrane system and their corresponding compartment, they do not appear to possess their own unique primary sequence signature. It is apparent, however, that these peptides all possess what may be called "consensus" structures (von Heijne, 1986; Tamm, 1991), which is to say they have certain patterns of properties which are common among many such peptides but it has been difficult to predict which sequences target particular membranes or their corresponding compartments. LSOs which target mitochondria, for example, can only be related generally by the fact that they are enriched in positively charged, hydroxylated, and hydrophobic amino acids. One solution to this enigma is that these peptides may be in possession of additional recognition properties which only emerge at a higher level of peptide organization such as at the secondary or tertiary level and perhaps are only manifest on or within the target membrane (Chupin et al., 1995; Golding and O'Shea, 1995).

† We are grateful to the British Technology Group Ltd., London, U.K., SmithKline Beecham, Betchworth, U.K., and the University of Essex Research Fund for support of this work.

* To whom all correspondence should be addressed.

‡ University of Essex.

§ Applied Photophysics Ltd.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

S0006-2960(96)00905-1 CCC: $12.00

There have been a number of studies of the structure of LSOs, particularly using CD, both in an aqueous solution and in the presence of phospholipid membranes or lipid micelles (von Heijne, 1986; Tamm, 1991). These studies have shown that there are indeed differences in the time-*average* secondary-structural chracteristics in the different environments. To date, however, no studies have been performed of the time-*dependent* transitions between such structures as revealed by CD which may also be correlated with either the membrane binding or insertion process. In the present study, we show that it is possible utilizing a newly developed, real-time fluorescence technique (Wall et al., 1995a) that both the membrane binding and insertion properties of the LSO of subunit IV of cytochrome *c* oxidase known as p25 (Maduke & Roise, 1993, Leenhouts et al., 1995) are available during the course of the same experiment (Golding & O'Shea, 1995; Wall et al., 1995a). Correlation of these data with structural changes, obtained with a stopped-flow CD technique, indicates that the LSO must first insert into the membrane in a random conformation and then adopts an intramembrane helical structure. It is thought that it is in this conformation that the LSO may "dock" with the putative membrane receptor before translocation takes place. Thus, it seems possible that a particular three-dimensional structure of the LSO within the membrane may be necessary in order to specify a unique membrane target.

MATERIALS AND METHODS

All chemical reagents were of the highest purity commercially available. Dipalmitoylphosphatidylethanolamine (DPPE), phosphatidylcholine (PC), and phosphatidylserine (PS) were obtained from Lipid Products (U.K.), and FPE was synthesized as previously described according to Wall et al. (1995a). p25, which is the leader sequence of the nuclear-encoded subunit IV of mammalian cytochrome *c* oxidase (EC 1.9.3.1), was synthesized and purified by HPLC by Dr. David Roise, University of Califronia, San Diego, and purchased in pure form from Peptide Products (U.K.). Monodisperse, 100 nm diameter unilamellar phospholipid vesicles of a well-defined lipid composition (of either 85% PC/14.5% PS/0.5% FPE or 99.5% PC/0.5% FPE) were prepared using a pressure-extrusion method as previously described and labeled solely in the outer-bilayer leaflet with FPE (Wall et al., 1995a).

Electrostatic Theory of the Membrane−Solution Interface. The effects of changes of the number of surface charges on the membrane−solution interface promote corresponding changes in the electrostatic potential on the membrane surface (Ψ) (McLaughlin, 1989; Wall et al., 1995b) which affects and is affected by concentrations of counterions (C) such as inorganic ions (including protons) on the membrane's surface according to the Boltzmann equation (eq 1):

$$\log(C_S/C_B) = 0.059\Psi \text{ volts} \quad (1)$$

where C = the concentration of protons in the bulk solution (B) and on the membrane surface (S), respectively.

In a related manner, it has been demonstrated that the location of any acid−base moiety such as fluorescein at the membrane−solution interface confers on the acidic group sensitivity to the electrical potential on the membrane surface (Wall et al., 1995a). Combination of the logarithmic form of the Boltzmann equation (eq 1) with the Henderson−Hasselbalch equation, therefore, leads to a dependence of the $pK_{apparent}$ of the acid/base group to the potential (Ψ) in the manner of eq 2.

$$pK_S = pK_B + \Psi \quad (2)$$

Thus, for an acidic group such as that of the fluorescein moiety, covalently bound to phosphatidylethanolamine (FPE) precisely located at a membrane surface, changes of the electrostatic surface potential at constant bulk phase pH will affect the protonation state of FPE, leading to changes of fluorescence. This property has been shown to offer the possibility of measuring the interactions of any factor which affects the electrical field experienced by the fluorescein moiety at the membrane surface. Charged molecules such as Ca^{2+}, peptides, and proteins, therefore, may be observed to interact with membranes in real time and with great sensitivity.

Time Resolution of p25 Interactions with FPE−Phospholipid Vesicles. Charged (PC/PS) and uncharged (PC) FPE-labeled phospholipid vesicles were prepared and subjected to fluorescence investigation with a single excitation wavelength of 490 nm, with emitted light recorded above 500 nm with an Applied Photophysics DX-17 MV (Leatherhead, U.K.) stopped-flow mixing apparatus equipped with an optical system suitable for fluorimetric recordings and a kelvinator temperature regulation device. The measured dead time of this apparatus was found to be 1 ms. The customized apparatus from the same company designed for both steady state spectral and time-resolved single-wavelength recordings of circular dichroism (CD) was utilized for studies of the p25 interactions with membranes.

Experiments performed at identical lipid and peptide concentrations inolving 90° light scattering (at 600 nm) indicated that phospholipid vesicle aggregation did not occur and compromise the interpretation. Similarly, CD spectra taken at lower concentrations of peptide did not cause any changes of the CD spectrum interpreted to indicate that peptide aggregation did not occur under the conditions of our experiments.

The resultant time courses of either fluorescence or CD signals were analyzed using either the proprietary software of the Applied Photophysics instruments, or the data were downloaded in the ASCII file format and then acquired by benchtop microcomputers with additional (but similar) software such as Ultrafit (Biosoft, U.S.A.). The various components of the data were best described by eq 3.

$$\text{observed signal} = (\text{initial signal-1} \times \exp k_1 t) + (\text{initial signal-2} \times \exp k_2 t) \quad (3)$$

Thus, the binding phase of p25 with membranes was characterized by two rate constants (k_1 and k_2), and the insertion phase was also characterized by two such rate constants. The time evolution of the CD signals following interaction with the membrane vesicles, however, was found to be best-described by a single-exponential process, yielding just one rate constant (k_1).

The total amplitudes of the fluorescence signals for both the binding and insertion phases plotted against the p25 concentrations were found to be sigmoidal. These data were

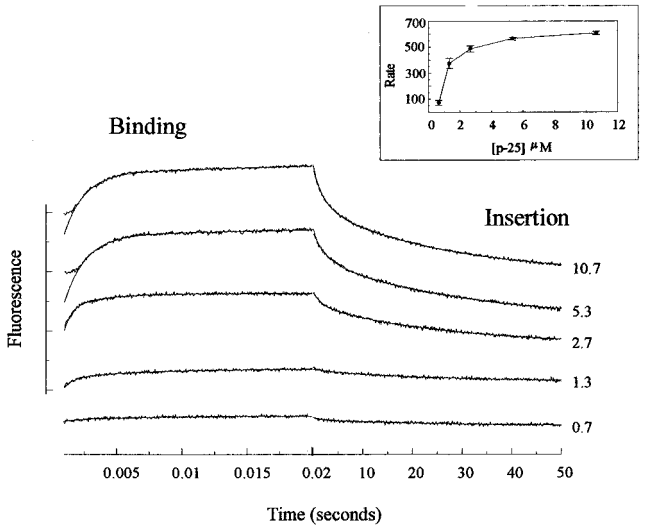

FIGURE 1: FPE-labeled phospholipid vesicles (213.5 μM PC) in 280 mM sucrose/10 mM Tris at pH 7.5 were stopped-flow mixed with the indicated concentrations (micromolar) of p25 in the same medium. Experimental traces covering two time periods (0−0.02 and 0.02−50 s) have been combined and are illustrated. Each trace represents the average of at least three independent experiments. The components of the traces labeled binding and insertion were independently fitted to eq 3. The resultant theoretical fits excluding the instrumental dead time and flow period have been drawn as solid lines through the data points. Experimental traces have been offset for clarity. The inset shows a plot of the rate constant k_1 binding (s^{-1}) against the corresponding concentration of p25. The error bars represent the standard error of the mean for each point shown.

analyzed according to eq 4 and yielded values for the Hill coefficient (n)

$$\text{observed signal} = (100\%\ \text{signal} \times [\text{p25}]^n)/(50\%\ \text{signal} + [\text{p25}]^n) \quad (4)$$

RESULTS

Interactions of p25 with FPE-Labeled Phospholipid Membranes. On mixing of FPE-labelled membranes with p25 complex, multiphasic, fluorescence changes were observed (see Figure 1). An initial rapid ($t_{1/2}$ of ca. 10 ms) process-(es) leads to an increase in fluorescence intensity, consistent with an increased electropositive surface potential caused by addition of positively charged p25. We assign this process to the binding of the oligopeptide to the membrane surface, therefore, and Figure 1 is labeled accordingly. This rapid binding reaction is followed by a slow decay ($t_{1/2} \sim 30$ s) of the fluorescence intensity, suggesting the "loss" of positive charge from the membrane surface. A plausible explanation for this reduction in fluorescence intensity is that, following association with the membrane, the peptide or its charged limbs insert into the interior of the membrane (see eq 2). Once immersed in the hydrophobic core of the membrane, these charges are effectively insulated from the electrical double layer of the membrane surface and do not further influence the protonation state of FPE or the surface potential upon which the latter depends.

Closer examination of the time courses shown in Figure 1 revealed that both the rapid initial process and the subsequent decay comprise two phases and may be fitted well to the sum of two exponentials, as given in eq 3. Depending on the circumstances, the rate constants for

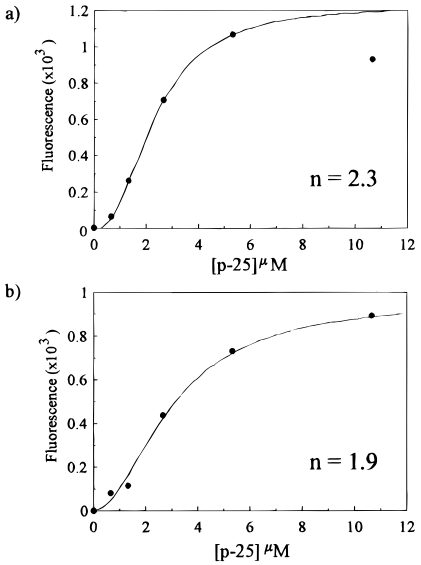

FIGURE 2: Effects of p25 concentration on the amplitude of the binding (a) and insertion (b) phases of the interaction of the peptide with the membrane. Fluorescence signals from studies such as those shown in Figure 1 were fitted to eq 4 which describes cooperative activity and yields a Hill coefficient (n). In the binding plot (a), the point at 10.7 μM p25 was not included in the cooperative fit as the extra charge added to the membrane was considered to affect the overall binding of the p25; on this basis, it was regarded as an outlier. Standard errors lie within the size of the point marker.

binding (k_1 and k_2) were found to be about 100−1000 times larger than those of the insertion process.

Figure 1 also reports the dependence of the fluoresence time courses on peptide concentration (from submicromolar to 11 μM) at constant lipid (PC) concentration. The experimental data have been plotted together with their fits to the rate equation (see eq 3). The fastest process displayed a clear dependence on peptide concentration below 4 mM, consistent with the assignment of this process to an initial binding event (inset to Figure 1). At higher p25 concentrations, however, this rate constant tends toward a limiting value indicating coupling of the binding reaction to a unimolecular process. The rate constants of the other components of both the binding and insertion phases defined by the rate equations were found to be independent of peptide concentration (not shown).

It is also apparent from Figure 1 that the amplitudes of the fluorescence signals of both the binding and insertion phases increase with increasing concentrations of the peptide. This is shown more clearly in Figure 2, where it may also be seen that the p25 concentration dependencies of the amplitudes of the binding and insertion processes are sigmoidal and are, perhaps, indicative of cooperative binding. Reasonably good "data fits" to an equation describing cooperativity (eq 4) were obtained (Figure 2) and yielded Hill coefficients of around 2. This is the first demonstration that the interaction of a free LSO with membranes may be cooperative. The appearance of cooperative phenomena indicates that the binding and insertion phases of the peptide−membrane interactions are more complex than has been previously supposed. One interpretation of this data, however, is that perhaps the initial membrane contacts involve interactions between two peptide molecules.

Biological membranes often possess a net negative surface charge, and as many signal/leader sequences possess a net positive charge, it is likely that the Coulombic attraction

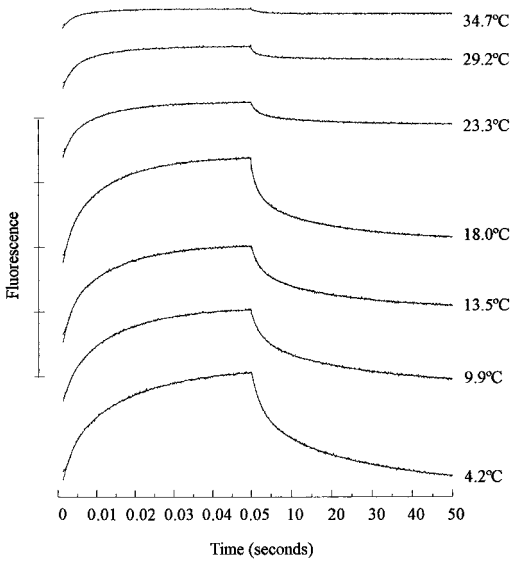

FIGURE 3: p25 (2 μm) was stopped-flow mixed with 213.5 μM FPE-labeled phospholipid vesicles at the indicated temperatures but otherwise as detailed in Figure 1.

between the peptides and the phospholipid head groups affects their mutual interaction. Studies designed to explore this relationship were undertaken by preparing membranes into which surface charges in the form of various concentrations of PS were introduced. The time courses of the fluorescence changes following rapid mixing of p25 (up to 5 μM) with such membranes (15% PS/84.5% PC) were qualitatively the same as those seen using uncharged membranes (Figure 1). The presence of the negative charge on the membrane surface, however, caused no appreciable changes of the respective rate constants.

The effects on the rates of binding and insertion of varying the lipid concentration at a constant p25 concentration were also studied. The rates and extent of binding were found to be dependent upon the concentration of membrane present in the reaction mixture. The rate constants of binding and insertion increased more or less linearly up to about 600 s^{-1} with the lipid concentration until an apparent saturation is reached at around 100 μM total phospholipid.

Determination of the Activation Energies for p25 Binding and Membrane Insertion. The effects of temperature on the interactions between p25 and the PC membrane system are shown in Figure 3. As the temperature was increased from 4 to 37 °C, the rates of binding and insertion increased but the amplitudes of the fluoresence changes associated with these reactions decreased. This behavior is consistent with rapid binding of p25 to the membrane surface in a reaction characterized, as is usual for binding processes, by a positive activation energy and a negative enthalpy. As binding is a prerequisite for insertion, elevated temperatures will decrease the extent of insertion by opposing formation of the membrane/peptide complex. An Arrhenius analysis of the data given in Figure 3 yielded activation energies for the respective components comprising binding and insertion which are reported in Table 1 together with those obtained using PC/PS membranes.

It is interesting that the activation energies for both binding processes to negatively charged membranes are much larger than those for binding to the uncharged membrane. The activation energy for k_1 of the p25 insertion reaction with the charged membranes, however, is larger than that found

Table 1: Activation Energies for Membrane Binding and Insertion[a]

membrane phospholipids	activation energy, k_1 (kJ K^{-1} mol^{-1})	activation energy, k_2 (kJ K^{-1} mol^{-1})
PC binding	6.4	15.6
PC insertion	12.7	27.8
PC/PS binding	45.5	27.2
PC/PS insertion	19.2	0

[a] Summary of the activation energies for the processes described by k_1 and k_2 from eq 3 for both binding and insertion reactions obtained by Arrhenius analysis of the data presented in Figure 3 and similar experiments with PC/PS phospholipid vesicles.

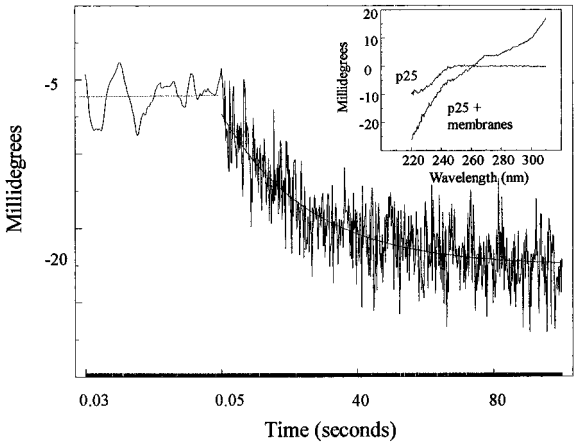

FIGURE 4: CD spectra (millidegrees versus wavelength) of 110 μM p25 in the absence and presence of phospholipid vesicles (900 μM PC) in 280 mM sucrose/10 mM Tris at pH 7.5 are shown in the inset. Spectra were measured from 220 to 310 nm over a 0.5 nm step and a 2 mm path length cell. The main figure shows the change of the CD signal at 225 nm over the time periods (0−0.05 and 0.05−100 s). The trace has been fitted to a single exponential process yielding a single rate constant of 0.04 s^{-1} drawn as a solid line through the experimental data points. Traces are the average of four data sets.

with the uncharged membranes but smaller for the k_2 process. At room temperature, however, there is little difference between the observed rates of binding and insertion. Thus, the respective temperature dependencies under varying surface charge, electrolyte concentration, etc., would reveal the relative contributions of the various thermodynamic factors involved in the binding and insertion processes.

Structural Changes of p25 during Interaction with Membranes. The time courses of structural transitions of p25 have been studied using stopped-flow CD methods. Because of a suitable choice of observation wavelength, the kinetics of secondary structural transitions may be followed and compared with those obtained from the fluorescence studies. In this way, it is possible to associate either membrane binding or insertion with conformational transitions of the peptide.

In order to select appropriate wavelengths at which to perform kinetic measurements, static CD spectra of p25 were recorded in the absence and presence of phospholipid membranes. The resulting CD spectra are shown in Figure 4 (inset). Each spectrum shown is an average of four independent scans and is corrected for intrinsic polarization of the instrument. The spectrum of p25 obtained in the presence of the phospholipid vesicles has also been corrected for any contribution made by the membranes in the absence of the peptide.

Figure 4 (inset) shows the CD spectrum of p25 in the sucrose-based aqueous medium. There was little dichroism

between 310 and 250 nm; below this wavelength, a trough develops at 220 nm. This spectrum is in good agreement with earlier studies (Tamm, 1991) and implies that p25 possesses a random structure when in an aqueous medium of low ionic strength. The p25 CD spectrum in the presence of the membrane preparation, however, is significantly different, exhibiting a CD signal of +16 millidegrees at 310 nm, a shoulder at 270 nm, an isodichroic point at 260 nm, and a strong negative feature (−25 millidegrees) at 220 nm. This spectral distribution is consistent with the adoption of a helical conformation by p25 on association with the membranes and is consistent with other studies (Tamm, 1991). At the concentrations employed in these studies, no evidence that the peptides became self-aggregated was found.

Stopped-flow CD revealed that on mixing p25 with phospholipid membranes no dichroic changes were observed over the first 50 ms at 225 nm. Over the next 30−50 s, however, a significant change in the CD signal was seen, consistent with helix formation as indicated in the spectrum shown in Figure 4 (inset), and which followed a single-exponential time course ($k = 0.04$ s^{-1}). Comparison with the rate constants for the binding and insertion phases of p25 clearly demonstrates that p25 membrane insertion is closely related to the appearance of secondary structure and the single CD rate constant was on the same order as the second exponential (k_2) for the p25 insertion process.

DISCUSSION

It has been established that localization of a fluorescent probe such as FPE at membrane−solution interfaces offers the possibility of making sensitive real-time measurements of the interactions of peptides or proteins with membranes in a virtually noninvasive manner (Wall et al., 1995a). This is well-illustrated by the stopped-flow mixing experiments reported here which reveal that on addition of the leader sequence, p25, to defined preparations of phospholipid membranes (Figure 1) clearly resolved flourescence time courses may be observed. These fluorescence changes are complex, comprising rapid flourescence enhancement followed by much slower quenching, corresponding to the multistep nature of the interactions between the peptide and membrane. At low peptide concentrations, the rate constant of the fastest transient displays a dependence on peptide concentration, and at [p25] $\sim$ 1 μM, the value of the rate constant k_1 is $\sim$400 s^{-1}. This strongly suggests that the most rapid process resolved in Figure 1 represents the initial binding reaction; if it does not, a preceding binding reaction must be postulated, having an association rate constant greater than 10^8 M^{-1} s^{-1}. This assignment is strongly supported by Figure 2a which relates the amplitude of the faster phase signals to p25 concentration and which has the general form of a binding reaction, saturating at high ligand concentrations. The slight reduction in the signal amplitude at the highest peptide concentration is thought to be due to the effects of the positively charged peptide on the membrane surface charge which slightly affects the peptide−membrane equilibrium. In addition, increasing temperature decreases this amplitude, in agreement with expectations based on the supposition that this amplitude reports the peptide/membrane complex concentration and that such binding reactions are generally characterized by negative enthalpy values (White & Wimley, 1994).

The initial fast process is itself complex, however, and does not comform to a simple second-order binding process. Figure 1 (inset) shows that the largest rate constant (k_1), which we assign to p25 binding to the membrane surface, first increases and then begins to saturate at concentrations of p25 greater than about 5 μM. It appears, therefore, that a simple collision-based mechanism in which an encounter between p25 and the membrane is all that is required for binding needs elaboration. An additional process coupled to and limiting the rate of binding would appear to be necessary to account for the approach of k_1 to a first-order rate limit ($\sim$600 s^{-1}, Figure 1 inset). This process could perhaps involve a rearrangement of the peptide following the initial membrane encounter which is required for fruitful binding. Alternatively, as FPE is sensitive to molecular rearrangements of membrane-bound proteins (Wall et al., 1995b), it is possible that simple collisional binding of p25 occurs which is then followed by a fairly rapid conformational change of the peptide disturbing the membrane surface electrostatic potential which is reported by FPE. Such rearrangements of the peptide may also be responsible for the second, p25 concentration-independent, rapid process (k_2 $\sim$ 20−60 s^{-1}) which leads to fluorescence enhancement.

The suggestion that p25−membrane interactions are more complex than previously reported is also emphasized by the cooperative nature of the concentration dependence of the extent of the interaction of p25 with the membrane, as shown in Figure 2. The Hill coefficient of the fit to the binding data is close to 2. One interpretation of this observation is that at least two molecules of p25 are involved in the process of binding to the membrane surface. These observations may have a significant bearing on our understanding of the mechanisms of interaction of LSO with membranes if it is considered that peptides interact with the lipid phase of the membrane prior to interaction with a receptor (Martoglio et al., 1995).

The slower processes following binding and which are associated with fluorescence quenching are most readily interpreted as insertion of the peptide into the membrane; thus, a proportion of the positive charge carried by p25 is lost from the membrane surface. The independence of the rate constants for these processes from p25 concentration is consistent with this interpretation. The fluorescence amplitude associated with insertion when plotted against p25 concentration (Figure 2) follows a sigmoidal curve (Hill coefficient of $\sim$2). This is possibly a consequence of the cooperative nature of the binding reaction (discussed above), the amplitude of the insertion phase being determined by the extent of binding; as this involves interactions between peptide molecules, so, apparently, would insertion. As with binding, however, it is possible that the insertion process involves significant conformational changes which may influence the membrane surface potential and/or that insertion is truly cooperative and thus may also involve interactions between at least two molecules of p25 for incorporation into the membrane.

The kinetics of the interaction between p25 and membranes monitored through time-resolved CD spectroscopy offers the possibility of correlating peptide structural changes with either membrane binding or insertion processes. A comparison of Figure 1 with Figure 4 indicates that there is little, if any, change in CD at 225 nm during membrane binding, i.e. in the first 50 ms of reaction. This is interpreted

to indicate that peptide binding does not require, nor is accompanied by, formation of a well-defined or regular structure which is CD active. In other words, and perhaps unsurprisingly, in order to bind, the peptide does not need to adopt a particular structural conformation. Following binding, it has been suggested above that the peptide may then undergo a rearrangement on the membrane surface, which is associated with the rate constant k_2 binding. The population of p25 molecules on the membrane surface prior to insertion does not appear to possess regular structures, and individual molecules may well, therefore, simply take up any one of a number of random structures.

Over a longer time period, clear changes of the CD signal, indicative of helix formation, are apparent. The rate constants for the single exponential which describes the change of the CD signal ($k = 0.04\ s^{-1}$) is very close to those observed for the slower phase of the insertion (k_2) of p25 into the membrane monitored by the fluorescence (Figure 1). This implies that the onset of the formation of an intramembrane helical structure follows membrane insertion of p25. Following binding, therefore, the peptide would appear to insert into the body of the membrane initially as a nonhelical/random structure. To date, many models of membrane protein insertion have suggested, however, that helix formation takes place on the membrane surface, due to the amphiphilic nature of such peptides (von Heijne, 1986; Tamm, 1991). Similarly, DeGrado (1993) emphasizes that some oligopeptides such as periodic structures of Leu-Lys will also adopt helical structures when sitting on an interface between a polar and a nonpolar medium. In these models, helix formation follows membrane binding and the insertion events then occur after helix formation on the membrane surface; clearly, our results suggest that such models may not be universally applicable and depend upon a number of factors.

The effects of temperature on the kinetics of p25 binding and insertion seen with both the PC and PC/PS membranes appear to be more complicated than anticipated simply on the basis of an enhanced Coulombic attraction between the membrane and the peptide. The activation energies for the interaction of p25 with the various membranes shown in Table 1, therefore, suggest that important structural changes are taking place during both membrane binding and insertion.

The activation energies of the binding and insertion processes are also revealing, although the multitude of intermolecular interactions involved in the initial binding which may contribute to the overall binding phenomenon (White & Wimley, 1994; Watts, 1995) will require further study. If we are correct in our assignment of the k_2 *insertion* process to the formation of an α-helix, however, then according to Table 1 we may state that it occurs with an activation energy of 27.8 kJ K^{-1} mol^{-1}. It is interesting, however, that this is within the range of values identified by White and Wimley (1994) for such a process.

The activation energies for binding of p25 to the surface of the PC membrane are just over 6 kJ mol^{-1} from k_1 and nearly 16 kJ mol^{-1} from k_2. The effect of incorporating an electronegative surface charge, however, significantly elevates the activation energy of the process represented by k_1 by a factor of approaching 8 to nearly 46 kJ mol^{-1}. Whereas the effect of the surface charge on the k_2 parameter of the binding reaction is not even doubled. Clearly, the presence of the extra negative charge on the membrane surface does not simply augment the binding of positively charged p25 by Coulombic means but gives credence to the possibility that additional processes are taking place which involve molecular rearrangements of the peptide.

The temperature dependence of the kinetics of the membrane insertion of p25 is equally complex. The values obtained for the kinetic components of the insertion phase shown in Table 1 indicate that k_1 and k_2 also may represent different phenomena. The activation energy of k_1 is half as large as that found for k_2. The most pronounced effect of the surface charge, however, is that the relatively large value for the activation energy of the k_2 insertion into the PC membrane is effectively insensitive to temperature in the PC/PS membrane system.

The studies described above have been directed toward understanding the nature of the interactions of a leader peptide with a phospholipid membrane. The LSO target *in vivo*, however, is thought to be a protein complex located within the mitochondrial membrane (Keibler et al., 1993; Stuart et al., 1994; Mayer et al., 1995). With the demonstration that membrane-located peptide channels may be openly accessible to the hydrophobic domains of the lipid membrane (Martoglio et al., 1995), the virtue of studying the interaction of LSOs with the simpler lipid phase is underlined because such events may prelude the interaction of such peptides with their receptor complex. In other words, given the large affinity of the leader/signal peptides for the lipid phase of membrane, it is a strong possibility that their target binding sites may be within the lipid core of the membrane or at least in addition to an extramembrane binding domain.

In light of the foregoing points, the question posed earlier in this paper concerning the level of structural organization of the peptide in which the targeting information lies may now be reconsidered. Thus, the membrane target receptor may well recognize both the intramembrane secondary structural characteristics of the LSO as well as the more polar/positively charged extramembrane structure, and it is likely that the positive charge disposition, in particular, is an important consideration. In other words, the primary amino acid sequence may reflect this added complexity for all the leader/signal peptides as the so-called consensus structures, but particular targeting information may reside in the conformation of the peptide within the hydrophobic core of the membrane and the positive charges outside of the membrane.

In addition to information concerning the interaction of peptides with membranes, the techniques described in the present paper offer a new means of studying a number of other membrane-based properties of proteins such as folding and insertion.

REFERENCES

Blobel, G., & Dobberstein, D. (1975) Transfer of proteins across membranes, *J. Cell Biol. 67*, 835−862.

Chupin, V., Leenhouts, J. M., de Kroon, A., & de Kruijff, B. (1995) Cardiolipin modulates the secondary structure of the presequence peptide of cytochrome oxidase subunit IV, *FEBS Lett. 373*, 239−244.

DeGrado, W. F. (1993) Catalytic molten globules, *Nature 365*, 488−489.

Gilmore, R., & Blobel, G. (1982) Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants, *Cell 42*, 497−505.

Golding, C., & O'Shea, P. (1995) The interactions of signal sequences with membranes, *Biochem. Soc. Symp. 23*, 971−976.
Keibler, M., Becker, K., Pfanner, N., & Neupert, W. (1993) Mitochondrial protein import - specific recognition and membrane translocation of preproteins, *J. Membr. Biol. 135*, 191−207.
Leenhouts, J. M., Torok, Z., Chupin, V., & de Kruijff, B. (1995) A molecular model for the specific cardiolipin-presequence interactions, *Biochem. Soc. Symp. 23*, 968−971.
Maduke, M., & Roise, D. (1993) The import of a mitochondrial presequence into protein-free phospholipid vesicles, *Science 260*, 364−367.
Martoglio, M., Hofmann, M. W., Brunner, J., & Dobberstein, B. (1995) The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally towards the bilayer, *Cell 81*, 207−214.
Mayer, A., Nargang, F. E., Neupert, W., & Lill, R. (1995) MOM22 is a receptor for mitochondrial targeting sequences and cooperates with MOM19, *EMBO J. 14*, 4204−4211.
McLaughlin, S. (1989) The electrostatic properties of membranes, *Ann. Rev. Biophys. Biophys. Chem. 18*, 113−136.
Stuart, R. A., Cyr, D. M., & Neupert, W. (1994) HSP70 in mitochondrial biogenesis, *Experientia 50*, 1002−1011.
Tamm, L. (1991) Membrane insertion and lateral mobility of synthetic amphiphilic signal peptides in lipid model membranes, *Biochim. Biophys. Acta 1071*, 123−148.
von Heijne, G. (1986) Mitochondrial targeting sequences may form amphiphilic helices, *EMBO. J. 5*, 1335−1342.
Wall, J., Golding, C., van Veen, M., & O'Shea, P. (1995a) The use of fluoresceinphosphatidylethanolamine as a real-time probe of peptide-membrane interactions, *Mol. Membr. Biol. 12*, 183−192.
Wall, J., Ayoub, F., & O'Shea, P. (1995b) The interactions of macromolecules with the mammalian cell surface, *J. Cell Sci. 108*, 2673−2682.
Watts, A. (1995) Biophysics of the membrane interface, *Biochem. Soc. Symp. 23*, 959−965.
White, S. H., & Wimley, W. C. (1994) Peptides in lipid bilayers - structural and thermodynamic basis for partitioning and folding, *Curr. Opin. Struct. Biol. 4*, 79−86.

BI960905I