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Interaction of a synthetic mitochondrial presequence with isolated yeast mitochondria: Mechanism of binding and kinetics of import

(protein translocation/amphiphilic peptides/membranes/*Saccharomyces cerevisiae*)

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ABSTRACT **The mechanism of interaction of a presequence with isolated yeast mitochondria was examined. A synthetic peptide corresponding to a matrix-targeting signal was covalently labeled with a fluorescent probe. Binding of the presequence to the surface of the mitochondria and translocation of the presequence into the interior of the mitochondria could then be monitored directly in solution by measuring changes in the steady-state fluorescence of the attached fluorophore. The binding step was rapid and reversible. Quantitation of the binding under equilibrium conditions suggested that the initial association of the presequence with the surface of the mitochondria occurred by partitioning of the presequence directly into the lipid bilayer of the outer membrane. Subsequent translocation of the bound presequence into the mitochondria was monitored by measuring the rate of disappearance of presequences sensitive to digestion by added trypsin. The efficiency of translocation was high, and the rate of the translocation was dependent on the electrical potential across the inner membrane. At physiological concentrations of presequence, the rate displayed first-order kinetics with respect to the concentration of bound presequence and had a rate constant of 0.19 min^{-1} at 20°C. Several kinetic models for the translocation of the presequence are presented that are consistent with the experimental results.**

The import of nuclear-encoded proteins into mitochondria requires that the proteins bind to the mitochondrial surface and subsequently be translocated across the membranes of the organelle. Experiments with gene fusions have demonstrated that the targeting sequences typically found at the amino termini of imported precursor proteins are solely responsible for the recognition of precursors by mitochondria, both *in vivo* and *in vitro* (1, 2). These import signals, termed presequences, display no common primary structure (3), but model studies with synthetic presequences have shown that the sequences are surface-active and have a strong affinity for model membranes (4). These studies suggested that presequences may interact directly with the lipid bilayer of the mitochondrial outer membrane.

The purpose of the current work was to use isolated, intact mitochondria in the quantitative analysis of the binding and import of a presequence and to determine the molecular interactions that are responsible for these events. Previous studies have demonstrated that radiolabeled synthetic presequences are capable of being imported into isolated mitochondria, but have not precisely quantified the interactions (5–8). The current study uses a fluorescently labeled synthetic presequence to demonstrate that a presequence can insert directly into the lipid bilayer of the mitochondrial outer membrane and is imported with kinetics that display first-order dependence on the concentration of the lipid-bound form of the presequence. The studies provide a quantitative method to analyze protein binding to and translocation across biological membranes.

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MATERIALS AND METHODS

Synthesis and Labeling of Peptides. A peptide having the sequence MLSLRQSIRFFKPATRTLCSSRYLL was synthesized and characterized as described (9). The single cysteine residue was labeled with 5-iodoacetamidofluorescein (Molecular Probes) in 50 mM Tris·HCl, pH 8.0/0.5 mM EDTA/25% (vol/vol) CH_3CN. The fluorescent peptide was purified as a single, sharp peak on a cation-exchange column (Mono S, Pharmacia) by using a gradient from 0.01 to 1.0 M ammonium acetate (pH 7.0) in 25% CH_3CN. Under these conditions, modified and unmodified peptides were completely resolved. Fractions containing the modified peptide were lyophilized and dissolved in aqueous 50% ethanol. Concentration of the peptide was determined by amino acid analysis. The peptide was found to contain a single label for every molecule, based on the extinction coefficient of fluorescein (ε_{490} = 75 $cm^{-1}\cdot mM^{-1}$ at pH > 7.0).

Discontinuous Assays of Peptide Import. Yeast mitochondria were purified from strain D273-10B, using the growth conditions and isolation procedures of Daum *et al.* (10). Mitochondrial protein concentrations were estimated by measuring the A_{280} of mitochondria dissolved in 1% SDS (ε_{280} = 2.1 $cm^2\cdot mg^{-1}$). To assay import of the presequence, duplicate samples containing mitochondria (100 μg) in 1 ml of standard buffer (0.6 M sorbitol/10 mM potassium phosphate, pH 7.4/1 mM ATP/2 mM $MgCl_2$/0.05% fatty acid-free bovine serum albumin) were placed on ice. Some of the samples were treated with valinomycin (1 μg/ml) to dissipate the membrane potential. Other samples were treated with 1,10-phenanthroline (1 mM) to inhibit an internal, chelator-sensitive presequence peptidase activity. The fluorescent peptide was added to a final concentration of 50 nM, and the samples were placed at 20°C for various times. Following the incubations, the samples were chilled, trypsin (50 μg) was added to one of the duplicates, and the tubes were spun at 14,000 × *g* for 3 min at 4°C to reisolate the mitochondria. The supernatants were removed and the pellets were resuspended in 1 ml of import buffer lacking albumin and ATP. The supernatants and resuspended pellets were treated with Triton X-100 (0.1%) and briefly dispersed in a bath sonifier. Samples that had not contained trypsin were then treated with trypsin (50 μg/ml) and left at room temperature until the presequence was completely digested. Fluorescence in each fraction was read on an SLM/Aminco SPF-500 fluorometer (excitation, 490 nm; emission, 525 nm; 5.0-nm bandpass).

Continuous, Spectroscopic Assays of Peptide Import. Conditions were the same as those for the discontinuous assays except that the fluorescence of each sample was monitored continuously. The assays were performed in 1 ml of standard buffer at 20°C in a quartz cuvette. Mitochondria were added at time zero. Valinomycin and 1,10-phenanthroline, when used, were added at 30 sec. Import was initiated by addition

of the labeled presequence at 1 min. At the end of the assay, trypsin (50 μg) was added to digest all unimported presequences.

RESULTS

A synthetic peptide that corresponds to the amino-terminal 25 residues of the yeast cytochrome oxidase subunit IV precursor was labeled with iodoacetamidofluorescein. After purification by cation exchange, the fluorescent presequence was used to measure binding of the presequence to isolated yeast mitochondria and subsequent import of the bound presequence into the mitochondria. The discontinuous binding and import assays are similar to those typically used to measure import of full-length, radiolabeled precursor proteins (11). Duplicate samples of mitochondria were incubated for various times with the labeled presequence. After treatment of one of the duplicates with trypsin to digest unimported presequences, mitochondria were reisolated by centrifugation, and the fluorescence in the pellets was measured after they were dissolved in detergent. In samples not treated with trypsin (Fig. 1, ○), the presequence showed a high level of binding to mitochondria that was relatively constant over time. Some decrease in binding was observed in samples not treated with 1,10-phenanthroline, due to partial digestion of the presequence by a metal-dependent protease (unpublished data; see also ref. 8). In contrast, the amount of presequence protected from trypsin in energized mitochondria was initially small but increased over the course of the assay (Fig. 1 *Left*, ●). Depolarization of the membrane with valinomycin significantly reduced the amount of presequence protected over time (Fig. 1 *Center*, ●) and showed that import of the presequence was potential-dependent. Treatment of mitochondria with 1,10-phenanthroline to block degradation of the presequence, however, did not affect the import (Fig. 1 *Right*, ●).

The discontinuous assays of presequence import demonstrated that the fluorescent presequence was competent for import and that presequence import was accelerated by the electrical potential of the mitochondrial inner membrane. However, these assays required that the mitochondria be reisolated and dissolved for each time point in order to separate and assay bound and free material. This additional step limits the accuracy of the experiments in determining kinetic values, particularly if the translocation is not completely blocked during the reisolation step or if the mitochondria are damaged during centrifugation and release presequences that have been imported. Observation of the fluorescence of the presequence directly, however, allowed the concentrations of the bound, free, and imported forms of the presequence to be monitored continuously during the incubations without the need to reisolate the mitochondria.

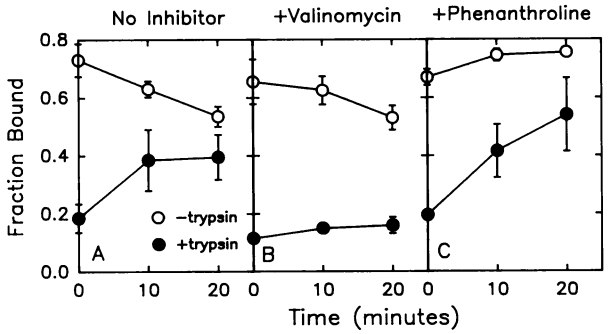

FIG. 1. Discontinuous assays of presequence import. Samples contained mitochondria (100 μg) suspended in 1 ml of standard buffer. Incubation mixtures contained the fluorescein-labeled synthetic presequence at 50 mM. The mitochondria were either untreated (*Left*) treated with valinomycin (1 μg/ml) (*Center*), or treated with 1,10-phenanthroline (1 nM) (*Right*). The samples were either treated with trypsin (●) or left untreated (○) prior to reisolation of the mitochondria. Fraction bound refers to the fraction of the total recovered fluorescence found in the resuspended mitochondrial pellet after dissolution with Triton X-100. The results were not corrected for rapidly reversible binding to the walls of the tubes, so that values greater than zero at the initial time points in the trypsin-treated samples are due to this background.

Binding of the fluorescent presequence to phospholipid model membranes decreases the fluorescence of the presequence. This quenching, which is rapid and occurs within the mixing time of the assay (<10 sec), is caused by the decreased absorbance of the fluorophore in the microenvironment of a membrane surface (S. T. Swanson and D.R., unpublished data). Quenching of the fluorescein-labeled presequence was also observed when the presequence was added to isolated mitochondria. The quenching was measured at various concentrations of both presequence and mitochondria to generate binding curves (Fig. 2). The observed curves are consistent with a two-state equilibrium between the free, unquenched presequence and a bound, quenched form of the presequence. Curves were fit to the data points by using the empirical equation $Q = Q_{max}C_M/(C_M^{50} + C_M)$, where Q is the observed quenching and C_M is the total concentration of mitochondrial protein (g/liter). The parameter Q_{max} is a constant obtained by extrapolating the quenching to infinite concentration of mitochondria. It corresponds to the quenching experienced by fully bound presequences. The parameter C_M^{50} is an empirical parameter that represents the mitochondrial concentration required for half-maximal quenching. At this concentration of mitochondria, half of the presequence in the sample is bound. The value of C_M^{50} was found to be independent of the concentration of the presequence under the conditions used.

The binding experiments showed that the mitochondria have a high capacity for binding of presequences. At a concentration of mitochondria equal to C_M^{50} (0.024 g/liter), the concentration of bound presequence, $[P_B]$, is one half the total concentration of presequence, $[P_T]$. Thus, for $[P_T] = 100$ nM, the ratio of bound presequence to total mitochondrial protein is 2.1 μmol/g. Since porin, by far the most abundant protein on the surface of the mitochondrial outer membrane, is present in significantly lower amounts [0.12 μmol/g in fungal mitochondria (12, 13)], there are insufficient outer

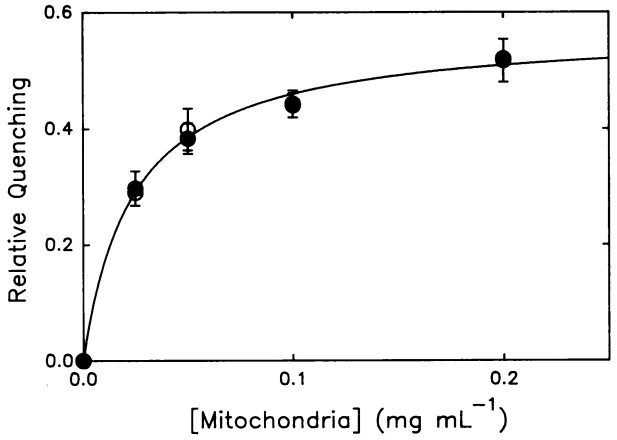

FIG. 2. Curves for binding of presequences. Conditions were the same as in Fig. 1, except that all buffers contained 1 mM 1,10-phenanthroline. Relative quenching is defined as $(F_T - F_{obs})/F_T$, where F_T is the fluorescence of the presequence added to 1 ml of a solution containing trypsin (50 μg) in the absence of mitochondria, and F_{obs} is the initial fluorescence of the presequence in assays containing various concentrations of mitochondria and lacking trypsin. Presequence concentration was 50 nM (○) or 100 nM (●). A curve defined by the equation $Q = Q_{max}C_M/(C_M^{50} + C_M)$ was fit to the points. The best value of Q_{max} was 0.57 and the best value of C_M^{50} was 0.024 g/liter for both presequence concentrations.

membrane proteins to account for the extent of presequence binding, and the presequence must, at least initially, interact directly with the lipid phase of the outer membrane. Although the behavior of synthetic amphiphilic sequences in model systems suggested that this type of interaction might be possible (9, 14–16), the results presented here demonstrate clearly that the binding of a presequence directly to lipids can occur in a biological membrane. Because of this, the binding can be treated as a simple partitioning process (17, 18), where the partition coefficient, Γ (liter/m^2), refers to the ratio between the concentration of bound presequence (with respect to the surface of the mitochondria) and the concentration of free presequence. The partition coefficient is proportional to $(C_M^{50})^{-1}$.

The kinetics of translocation of the presequence into mitochondria could also be measured with spectroscopic assays using the fluorescent presequence. Treatment of mitochondria with 1,10-phenanthroline inhibits the digestion of the imported form of the presequence within the mitochondria but does not affect the import process (Fig. 1*C*). Observation of the fluorescence of the labeled presequence under these conditions revealed that the quenched fluorescence of the bound presequence remained constant during the course of the assay (Fig. 3), presumably because the imported form of the presequence remained bound to a membrane surface within the mitochondria. Addition of trypsin at various times during the assay, however, resulted in the rapid increase of observed fluorescence as the external presequences were digested by the protease and the bound fluorescein was released from the surface of the outer membrane. The size of this increase could be used to calculate the concentration of presequence remaining untranslocated on the mitochondrial surface at any time during the reaction, and the kinetics of the translocation could thus be followed.

A series of import reactions using mitochondria treated with 1,10-phenanthroline were stopped with excess trypsin at various times (Fig. 3). These assays revealed a significant decrease in the amount of fluorescence released by the trypsin as the incubations progressed and the presequence was imported. The concentration of presequence bound to the surface of the mitochondria at each time was calculated using information obtained from the binding experiments. The concentration of presequence bound at the initial time is given by $[P_B]_0 = [P_T]Q_0(Q_{max})^{-1}$, where Q_0 is the initial quenching. The bound concentration at later times was calculated from the increase in fluorescence following trypsin treatment (ΔF). Since the digestion by trypsin is rapid relative to the import reaction (see Fig. 3) and since the relative molar fluorescence intensities of the free and digested forms of the presequence are the same ($I_F = I_D$), it can be shown that the change in fluorescence upon trypsin treatment is given by $\Delta F = (I_F - I_B)[P_B]$. This equation was rearranged and used to calculate $[P_B]$. The equation was put in terms of the total fluorescence of the presequence in the absence of mitochondria ($F_T = I_F[P_T]$) and the quenching of the fully bound presequence ($Q_{max} = (I_F - I_B)/I_F$), so that $[P_B] = [P_T]\Delta F/(Q_{max}F_T)$. Values of $[P_B]$ at the initial time and at several later times were determined from fluorescence measurements for a series of import experiments performed under energizing conditions in the presence of 1 mM 1,10-phenanthroline (Fig. 4). The results presented are for two different total concentrations of the presequence at several concentrations of mitochondria.

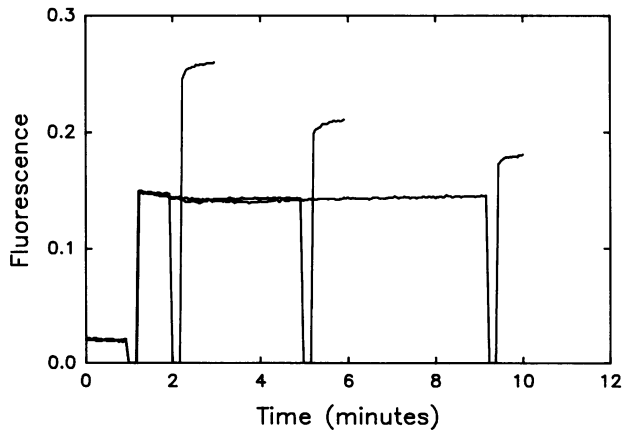

FIG. 3. Time course of protection of the presequence from trypsin in mitochondria treated with 1 mM 1,10-phenanthroline. Three separate experiments are superimposed in the figure. Mitochondria (200 μg) were suspended in standard buffer with 1 mM ATP. At 1 min, the fluorescent presequence was added to 50 nM. The reactions were quenched with trypsin (50 μg) at various times during the experiments. Breaks in the traces indicate times of the additions. The units of the fluorescence are arbitrary.

Kinetic Model for Presequence Import. The data from Fig. 4 have been fit using a kinetic model illustrated in Fig. 5. This model includes an initial, rapidly established partitioning between presequence free in the external solution (P_F) and presequence bound externally to the outer membrane of the mitochondria (P_B). This binding remains in equilibrium during the import and is described by the partition coefficient, Γ. After the presequence binds to the mitochondrial surface, its subsequent translocation can be considered to be analogous to a unireactant enzymatically catalyzed process, except that all the steps take place within the confines of the mitochondrial membranes. Because the overall reaction occurs between two phases, it is important to distinguish between concentrations that are relative to the volume of the bulk solution and those that are relative to the surface area of the mitochondrial phase. In the subsequent analysis, the concentrations of bound presequences or of intrinsic, membrane-bound proteins that are defined relative to the area of the external mitochondrial surface will be noted by a subscript or superscript S (for example, $[P_B]_S$). The concentrations ex-

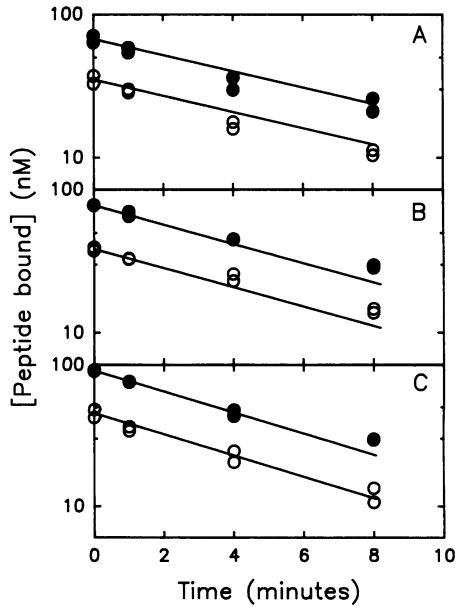

FIG. 4. Kinetics of the import of the presequence. The time course of protection from trypsin was monitored as described in the text. Presequence concentration was 50 nM (○) or 100 nM (●). Mitochondria were at 0.05 g/liter (*A*), 0.1 g/liter (*B*), or 0.2 g/liter (*C*). Lines were generated from the simplified form of the integrated rate equation (Eq. 2) with $(k_2K_E/K_S)/K_m^S = 0.19$ min^{-1} and $C_M^{50} = 0.024$ g/liter. The ordinate is plotted on a logarithmic scale.

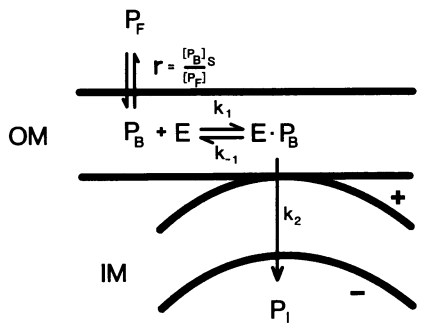

FIG. 5. Kinetic mechanism for the translocation of a presequence. OM, outer membrane; IM, inner membrane; E and $E \cdot P_B$, the unoccupied form of a catalyst of translocation and that catalyst occupied by the presequence, respectively. The partition coefficient, Γ, is defined in the text. The term P_I represents all imported forms of the presequence. If the peptidase in the matrix is active, the imported presequence is rapidly digested. When the mitochondria are treated with 1,10-phenanthroline to inhibit the peptidase, most of the imported presequence appears to remain membrane-associated on the extracytoplasmic side of the mitochondrial membranes.

pressed in the two different units are directly related to each other by a factor $K_S C_M$, so that $[P_B]_S = [P_B]/K_S C_M$. The value C_M is the concentration of mitochondria (g/liter), and K_S (m^2/g) is a proportionality factor that relates the surface area of the outer membrane of the mitochondria to the amount of mitochondrial protein.

The letter E in the scheme (Fig. 5) describes the unoccupied form of a translocation catalyst, referred to as the translocator, and the designation $E \cdot P_B$ refers to a complex between the translocator and the presequence. In order to formulate an overall rate equation, one must derive an expression for the concentration of $E \cdot P_B$. From the Briggs–Haldane steady-state assumption (19), $K_m^S = [E]_S[P_B]_S/[E \cdot P_B]_S = (k_{-1} + k_2)/k_1$. The parameter K_m^S is a pseudoequilibrium binding constant for the translocator–presequence interaction when the concentrations are expressed relative to the external surface area of the mitochondria. Since $[E]_S/[E \cdot P_B]_S = [E]/[E \cdot P_B]$, one can substitute and solve for [E], the bulk molar concentration of the unoccupied translocator: $[E] = K_m^S[E \cdot P_B]/[P_B]_S$. From conservation of mass, $[E]_T = [E] + [E \cdot P_B]$, where $[E]_T$ is the total bulk molar concentration of the translocator. Substitution and rearrangement gives $[E \cdot P_B] = [E]_T[P_B]_S/(K_m^S + [P_B]_S)$. The bulk molar concentration of the translocator, $[E]_T$, is related to the bulk concentration of mitochondria by an unknown constant of proportionality, K_E (mol of translocator per g of mitochondrial protein), such that $[E]_T = K_E C_M$. Substitution of this into the equation and conversion of $[P_B]_S$ to the bulk molar concentration of bound presequence gives $[E \cdot P_B] = K_E C_M[P_B]/(K_m^S C_M K_S + [P_B])$.

The appropriate rate equation for the measured value of presequence import is $d([P_F] + [P_B])/dt = -k_2[E \cdot P_B]$. The term $d([P_F] + [P_B])/dt$ is the instantaneous rate of change of the total external molar concentration of the presequence with respect to time. To fit the experimentally determined values of $[P_B]$ as a function of time, an integrated form of this equation was derived. From the binding experiments, the relationship between $[P_F]$ and $[P_B]$ is known, and $[P_F] + [P_B] = [P_B][(C_M^{50}/C_M) + 1]$. Substitution of this expression and the expression derived above for $[E \cdot P_B]$ into the rate equation and rearrangement gives $C_M K_m^S K_S d[P_B]/[P_B] + d[P_B] = -k_2 K_E (C_M)^2 (C_M^{50} + C_M)^{-1} dt$. Integration of this expression over the range $[P_B]_0$ to $[P_B]$ yields

$$C_M K_m^S K_S \ln([P_B]_0/[P_B]) + ([P_B]_0 - [P_B]) = k_2 K_E (C_M)^2 t/(C_M^{50} + C_M). \quad [1]$$

The data in Fig. 4 were fit to Eq. 1, with $C_M^{50} = 0.024$ g/liter and with the terms $K_m^S K_S$ and $k_2 K_E$ replaced by single constants. No unique convergent fit could be obtained, although the ratio of $k_2 K_E$ to $K_m^S K_S$ was constant and equal to 0.19 min^{-1}. One explanation for this lack of a unique fit is that the concentration of the surface-bound form of the presequence, P_B, was below the K_m^S value for the binding step and the translocator was not significantly occupied. This could normally be tested experimentally by using higher total presequence concentrations to increase $[P_B]_S$. Higher total presequence concentrations, however, cause increased mitochondrial respiration (see ref. 9) and could result in a decreased membrane potential or other artifacts.

As an alternative to increasing the concentration of the presequence in the experiments, the same kinetic data could be applied to a simplified version of the integrated rate equation that was appropriate for subsaturating P_B. In that case, $[E] \approx [E]_T$, and $[E \cdot P_B]$ could be neglected in the conservation-of-mass relationship. With this change, the derivation was continued as above and yielded the integrated form of the rate equation for subsaturating levels of presequence:

$$K_m^S K_S \ln([P_B]_0/[P_B]) = k_2 K_E C_M t(C_M^{50} + C_M). \quad [2]$$

It should be noted that Eq. 2 is the limit of Eq. 1 when $C_M K_m^S K_S \ln([P_B]_0/[P_B]) >> ([P_B]_0 - [P_B])$. The data from the presequence imports gave a good fit to this equation with $k_2 K_E (K_m^S K_S)^{-1} = 0.19$ min^{-1}. The lines shown in Fig. 4 were generated using this rate constant at appropriate values of $[P_B]_0$.

DISCUSSION

The use of a fluorescently labeled peptide with a sequence identical to a naturally occurring presequence that targets a protein to the mitochondrial matrix has facilitated the physical analysis of the interactions between such presequences and intact mitochondria. The three major findings of this work are that the presequence can bind directly to the surface of the mitochondrial outer membrane, that the import rate of the presequence is directly proportional to the concentration of the presequence on the mitochondrial surface, and that, at least within the concentration range tested, the transporter does not display saturation by the bound presequence.

The ability of presequences to bind directly to the surface of the mitochondrial outer membrane raises questions about the molecular basis for the specific recognition of precursors by mitochondria *in vivo*. Because the presequence is translated in the cytoplasm, there should be nothing to prevent it from inserting into any membrane that contacts the cytoplasm. The results presented here, however, suggest that this insertion would be rapidly reversible and that precursors incorrectly bound to other membranes would eventually find their way to the mitochondria. Although few studies have directly addressed the question of mitochondrial protein sorting *in vivo*, the possibility of transient incorrect binding of precursors to other membranes should not be ignored. The ability of presequences to bind to membranes also suggests that the role played by receptors for imported proteins (20–22) may involve interaction with the precursor after its initial association with the mitochondria or that these proteins may interact with another region of the precursor.

The observed kinetics of import of the presequence are consistent with a simple kinetic model for translocation (Fig. 5). Within the context of this model, there are at least two reasonable explanations for the observation that the rate of import shows first-order kinetics with respect to the concentration of bound presequence and does not saturate. Both are based on precedents established for the kinetic description of unireactant enzyme-catalyzed reactions. In the first expla-

nation, the Michaelis constant for the bound presequence, K_m^S, is higher than the concentration of presequence that can be used experimentally without damaging the mitochondria. Thus, the rate of import of the presequence is linearly related to the concentration of bound presequence in the range of concentrations that are experimentally accessible. This situation is analogous to an enzymatic reaction at relatively low concentrations of substrate, where the rate of the reaction is described by an apparent first-order rate constant, $k_{app} = V_{max}/K_m$, and $v = V_{max}[S]/K_m$, where [S] is the substrate concentration. For the mitochondrial case, $(k_2K_E/K_S)/K_m^S$ is equivalent to V_{max}/K_m. The value K_E/K_S is the total concentration of the translocator on the mitochondrial surface (mol of translocator per m^2 of outer membrane surface) and is a constant for any particular mitochondrial preparation. The expression k_2K_E/K_S is analogous to $k_{cat}[E]_T$ for an enzymatic reaction, where k_{cat} is the turnover number and $[E]_T$ is the total concentration of enzyme.

In the second explanation, the rate of the reaction is limited by two-dimensional diffusion of the bound presequence along the mitochondrial surface to the translocation site. This situation is analogous to the case of an enzyme reaction that is limited by the rate of diffusional contact between the enzyme and its substrate and occurs with enzymes that have highly efficient catalytic steps (23, 24). The effect of this on the kinetic scheme is that $k_{-1} << k_2$ (see Fig. 5) and the pseudoequilibrium binding constant becomes $K_m^S = k_2/k_1^S$. If one substitutes this into Eq. 2, the observed first-order rate constant becomes $k_1^SK_E/K_S$. Again, K_E/K_S corresponds to the concentration of the translocator relative to the mitochondrial surface.

An additional possibility that is not shown in the scheme in Fig. 5 but that cannot be excluded based on the kinetic measurements is that the import of the presequence is uncatalyzed; that is, translocation proceeds directly from the form of the presequence bound to the lipids on the surface of the mitochondria. In this case, the measured rate constant simply describes this first-order process. It should be emphasized that none of the three possibilities described here can be distinguished on the basis of the kinetic data in Fig. 4, nor can it be concluded that there are no other models consistent with the kinetics. The results do, however, provide a framework for the design of additional experiments to distinguish the various mechanisms.

As a final point, the kinetics observed for presequence translocation may be interesting from the standpoint of cellular physiology. Enzymes are thought to be under evolutionary pressure to increase their values of k_{cat}/K_m until their reaction rates become limited by diffusional association of an enzyme and its substate (23, 24). At the same time, the values of K_m must remain high enough that the enzymes are not saturated by substrate under normal physiological conditions. The rate-limiting step in the import of proteins into mitochondria *in vivo* is unknown. Translocation of the presequence, however, must precede the uptake of the rest of the precursor protein and is likely to contribute to the observed kinetics of import. The results presented here demonstrate that the rate of presequence translocation into isolated mitochondria does not saturate even at concentrations of presequence likely to be higher than those present in the cytoplasm (25). If the same is true *in vivo*, and the import system is not saturated by precursors, the rate of protein import into mitochondria would be proportional to the concentration of precursor proteins in the cytoplasm and could respond to changes in those concentrations. Thus, the net flux of precursors into mitochondria could be controlled directly by the nucleus at the level of transcription and translation rather than remotely by the mitochondria. Additional control of the import flux could also result from changes in the mitochondrial membrane potential, if the potential is coupled to the rate-limiting step of translocation. The models presented here should provide a useful starting point to understand the molecular and kinetic mechanisms of the translocation of presequences into mitochondria and ultimately to describe the overall process of protein import.

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