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Internal dynamics of lactose permease

(fluorescence anisotropy decay/orientational fluctuations/fluctuation–function relationship/transport protein)

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ABSTRACT **The transport protein lactose permease was reconstituted in vesicles of dimyristoylphosphatidylcholine, and the internal dynamics were studied by measuring the fluorescence anisotropy decay of the tryptophan residues and of a covalently bound pyrene label. For the tryptophans three relaxation processes and for the pyrene two relaxation processes with relaxation times in the nanosecond range were observed. The slowest process, of ≈50 ns, is assigned to orientational fluctuations of membrane-spanning helices. When the temperature is decreased below the lipid-phase transition, this relaxation process is slowed down and restricted in amplitude. Because the transport rate is known to also decrease below the phase transition, this observation suggests a coupling between internal dynamics and transport. This coupling is analyzed on the basis of the Kramers relation for chemical reactions.**

Over the past 20 years it has become clear that proteins are not static structures but instead are dynamic entities that undergo conformational changes in fulfillment of their function. Such conformational changes may occur upon binding of substrate or in an ensuing catalytic process. For a membrane protein involved in transport, binding with its conformational change is followed by the transport step, which requires another conformational change. The characteristic times of such conformational changes are given by the protein–substrate association rate or the catalytic turnover number, respectively, and range from below milliseconds to seconds.

In addition to these large and slow conformational changes proteins are known to undergo small and fast conformational fluctuations. Each conformational state in the above sense consists of a large number of substates, and transitions between substates occur as fluctuations with characteristic times extending from picoseconds to microseconds. The existence of substates and fluctuations has been demonstrated by a number of different techniques (for review, see ref. 1).

What is still under debate is the relevance of such fluctuations to protein function. Are they just random unavoidable thermal motions of the protein or are they correlated in a specific way to the slow conformational changes observed in substrate binding and catalytic processes? A model has been proposed in which the fluctuations and conformational changes obey a hierarchical order (2): the fastest fluctuations give rise to slightly slower ones, which in turn give rise to still slower ones and so on until, finally, the slowest fluctuations lead to a conformational change. In an attempt to give an experimental answer to the above question we investigated the fluctuations of the transport protein lactose permease (LPase) under conditions where the protein is known to be functionally either active or inactive.

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LPase is a protein of the cytoplasmic membrane of *Escherichia coli*, which catalyzes the transport of galactosides in symport with a proton across the membrane (for reviews, see refs. 3 and 4). The protein consists of 417 amino acid residues and acts as a monomer. Based on spectroscopic data and structural predictions, a model for the folding of LPase within the membrane was proposed: ten membrane-spanning α-helices form a ring the interior of which is filled with relatively hydrophilic amino acid residues suited to provide the sugar-binding site (5, 6).

To investigate a putative correlation between internal fluctuations and function, a membrane protein offers two distinct advantages over soluble proteins. (*i*) The activity of the membrane protein can easily be switched on and off by passing through the phase transition of the surrounding lipids (7–9). (*ii*) On the time scale of nanoseconds relevant for internal fluctuations, a membrane protein is immobilized, whereas a soluble protein undergoes rotational diffusion that renders detection of such internal fluctuations difficult, if not impossible.

The technique we used to study internal fluctuations is fluorescence anisotropy decay (FAD) (for review, see ref. 10). This technique permits detection of orientational fluctuations of intrinsic or extrinsic fluorophores with characteristic times in the range of picoseconds and nanoseconds.

MATERIALS AND METHODS

Sample Preparation. For measurements of the tryptophan fluorescence of LPase, cytoplasmic membrane vesicles were prepared from the permease-overproducing strain *E. coli* T206 (11) and used directly for purification and reconstitution of LPase. Control samples were prepared identically from the permease-deficient strain *E. coli* T184. For measurements on pyrene-labeled permease, cytoplasmic membranes vesicles of strain T206 were treated with *N*-ethylmaleimide (MalNEt) in the presence of substrate and subsequently incubated with *N*-(1-pyrenyl)maleimide (MalNPyr), leading to predominant labeling of Cys-148 (12). Control samples were prepared by preincubation of the samples with MalNEt without substrate, so that subsequent incubation with MalNPyr leads to labeling of cysteine residues other than Cys-148 (5); the most probable candidates are Cys-154 and Cys-333 (13, 14).

The samples and control samples of unlabeled and MalNPyr-labeled permease were purified and reconstituted into vesicles of dimyristoylphosphatidylcholine ([Myr_2]PtdCho) as described (15) with modification (5). The molar permease/lipid ratio was 1:1500 for unlabeled permease and 1:2500 for MalNPyr-labeled permease. Absolute concentrations of

Abbreviations: FAD, fluorescence anisotropy decay; LPase, lactose permease; [Myr_2]PtdCho, dimyristoylphosphatidylcholine; DodOMalt, dodecyl-*O*-β-D-maltoside; MalNEt, *N*-ethylmaleimide; MalNPyr, *N*-(1-pyrenyl)-maleimide; MalNPyr-ME, 2-(2-hydroxyethylsulfuryl)-*N*-(1-pyrenyl)succinimide; RES_s, residuals of the sum; RES_d, residuals of the difference.

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LPase in the two cases were ≈1 μM in 5 mM potassium phosphate buffer (pH 7.5) and ≈0.2 μM in the same buffer.

FAD Measurements. The apparatus has been described by Best *et al.* (16). For measurements of the tryptophan fluorescence, we used wavelength of excitation (λ_{ex}) = 300 nm and wavelength of emission (λ_{em}) = 365 nm. The intensity of the control samples without LPase was 5–8% of the intensity of the samples. For the MalNPyr fluorescence, we used λ_{ex} = 345 nm and λ_{em} = 375 nm, and the intensity of the control samples where Cys-148 was not labeled with MalNPyr was ≈25% of the intensity of the samples.

The fluorescence intensities $I_{\parallel}(t)$ and $I_{\perp}(t)$ of the MalNPyr samples were first corrected for background by subtracting the corresponding intensities of the control samples to extract the signal of MalNPyr bound specifically to Cys-148 (Pyr-Cys-148). Analysis of the control sample yielded the signal of MalNPyr bound to cysteine residues other than Cys-148 (Pyr-Cys-*X*). From the corrected intensities $I^c_{\parallel,\perp}(t)$, the total intensity was constructed as $S(t) = I^c_{\parallel}(t) + 2I^c_{\perp}(t)$ and the anisotropy as $R(t) = D(t)/S(t)$ with $D(t) = I^c_{\parallel}(t) - I^c_{\perp}(t)$. Analytical curves for the sum and the anisotropy were assumed as $s(t) = \Sigma\, a_i \exp(-t/\tau_i)$ and $r(t) = \Sigma\, b_j \exp(-t/\phi_j) + r_\infty$, and $s(t)$ and $d(t) = s(t)\cdot r(t)$ were fitted, after convolution with a fluorescence standard, to $S(t)$ and $D(t)$ (16).

The goodness of the fits was examined by inspection of the residuals of the sum, $RES_s(t)$, and of the difference, $RES_d(t)$, and by the value of χ^2 (16). The residuals of the sum show oscillations with a period of ≈3 ns, which are, however, a common artifact of single-photon counting electronics (17, 18).

Relaxation Time for Rotational Diffusion of a Membrane-Spanning Helix. A membrane-spanning helix performing orientational fluctuations is described as a symmetric ellipsoid undergoing rotational diffusion in an ordering potential. A fluorophore on the ellipsoid has a fixed orientation. The fluorescence anisotropy decay of such an ellipsoid, in the limit of high order, is monoexponential with the relaxation time (19)

$$\phi = \frac{\langle \vartheta^2 \rangle}{2D_{\perp}}. \qquad [1]$$

Here, $\langle \vartheta^2 \rangle$ denotes the mean-square fluctuations of the angle ϑ of the long axis of the ellipsoid relative to its mean orientation, and $D_{\perp}$ denotes the coefficient for rotational diffusion about a short axis. $D_{\perp}$ can be obtained from the relation $D_{\perp} = 3k_BT/(16\pi\eta c_1^3)[2\ln(2c_1/c_2) - 1]$ with c_1 and c_2 denoting the long and short half axes of the ellipsoid and η denoting the viscosity of the surrounding medium (20). Using $2c_1 = 30$ Å and $2c_2 = 10$ Å together with $\eta = 4p$ as determined for rotational diffusion of a membrane protein about its long axis (21), one obtains for a membrane-spanning helix $D_{\perp} = 5 \times 10^5 \cdot \text{sec}^{-1}$.

The value of $\langle \vartheta^2 \rangle$ can be obtained from experiment. The relaxation amplitude b and the residual anisotropy r_∞ yield the orientational order parameter as $S = [r_\infty/(b + r_\infty)]^{1/2}$ (22). It is defined as $S = \langle (3\cos^2\vartheta - 1)/2 \rangle$, which in the limit of high order or small values of ϑ can be expanded in powers of ϑ leading to

$$\langle \vartheta^2 \rangle = \frac{2}{3}(1 - S). \qquad [2]$$

The Kramers Relation for Rotational Diffusion. If a reaction requires a transition between two states separated by an energy barrier ΔG, the reaction rate k can be described by the Arrhenius relation $k = k'\exp(-\Delta G/RT)$. Explicit expressions for the factor k' have been derived by Kramers (23) modeling the reaction as a Brownian motion of a particle along the energy profile. For high viscosity, he obtained $k' = 2\pi m\nu_o^2/\gamma$ with m denoting the mass of the particle, ν_o the oscillation frequency in the energy well, and γ the friction coefficient. For simplicity, we have assumed that the curvature of the energy profile in the well is the same as at the top of the barrier. By using the Einstein relation $D_t = k_BT/\gamma$ for the coefficient of translational diffusion D_t and the equipartition theorem $m(2\pi\nu_o)^2\langle x^2 \rangle/2 = k_BT/2$, with $\langle x^2 \rangle$ denoting the mean-square fluctuations of the position of the particle, the Kramers relation becomes $k' = D_t/(2\pi\langle x^2 \rangle)$. In this form, it can easily be adapted to reactions controlled by rotational diffusion leading to $k' = D_{\perp}/(2\pi\langle \vartheta^2 \rangle)$. If Eq. 1 for the relaxation time ϕ of a symmetric ellipsoid is employed, one obtains

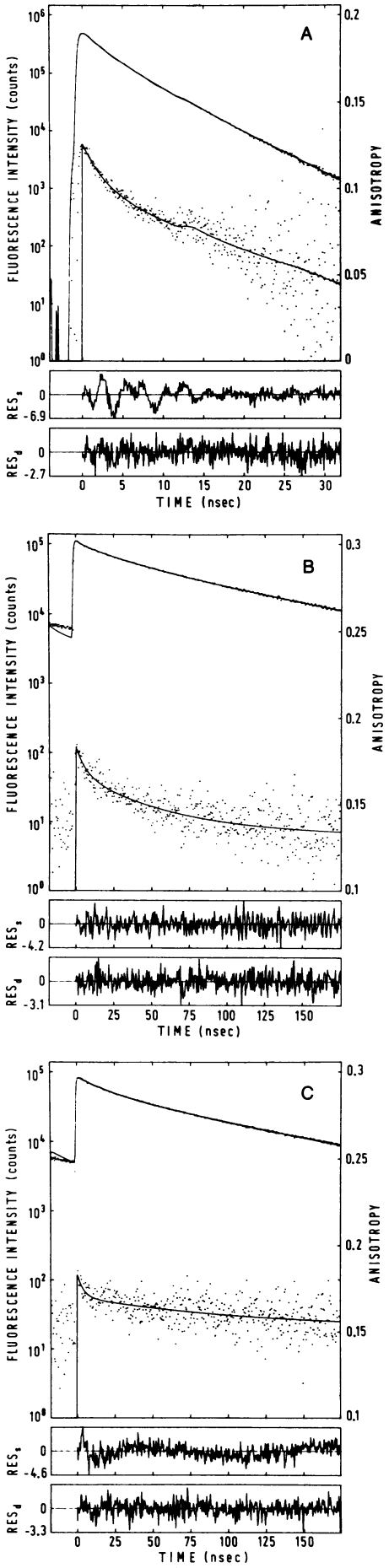
$$k' = 1/(4\pi\phi). \qquad [3]$$

RESULTS

In a first series of experiments we used the tryptophan fluorescence of LPase to investigate the orientational fluctuations of the 6 tryptophan residues. For that purpose, LPase was purified and reconstituted into vesicles of [Myr_2]PtdCho. In this lipid, LPase has been shown to be fully active at temperatures above the lipid phase transition temperature at T_t = 28°C (28). The fluorescence intensity and anisotropy decay at 28°C is shown in Fig. 1*A* for a time window of 30 ns. The intensity could be fitted by a double-exponential decay with the lifetimes $\tau_1^{Trp} = 1.4 \pm 0.5$ nsec and $\tau_2^{Trp} = 5.0 \pm 0.5$ nsec, and the relative amplitudes $a_1^{Trp} = 0.40 \pm 0.05$ and $a_2^{Trp} = 0.60 \pm 0.05$. These values are typical for tryptophan residues in proteins (10). The anisotropy decay is fast at short times and continues more slowly at longer times. The fast relaxation was analyzed by restricting the time window to 12 ns. A fit by two exponentials and a constant term yielded the results given in Table 1. They lead to an initial anisotropy of $r_0 = 0.132$, a value that lies within the wide range of initial anisotropies measured for tryptophan residues in proteins (10). The value of r_∞ is irrelevant, as it is a consequence of the narrow time window. The slow relaxation could not be analyzed accurately due to scatter of the data and lack of a limiting value for the anisotropy. Lower and upper limits for the relaxation time ϕ_3^{Trp} were obtained as 20 and 100 nsec.

To investigate the slow relaxation in more detail, LPase was labeled with MalNPyr, which has a lifetime of ≈100 nsec. The fluorescence intensity and anisotropy decay of such a MalNPyr label bound to Cys-148 of LPase is shown in Fig. 1*B*. A fit of the intensity yielded the lifetimes $\tau_1^{Pyr} = 22 \pm 0.5$ nsec and $\tau_2^{Pyr} = 109 \pm 0.5$ nsec with the relative amplitudes $a_1^{Pyr} = 0.28 \pm 0.05$ and $a_2^{Pyr} = 0.72 \pm 0.05$. These values again agree with those from the literature (24). The results of a fit of the anisotropy by two exponentials and a constant term are given in Table 1. Here, the initial anisotropy becomes $r_0 = 0.188$, which again is compatible with results in the literature (25). If the anisotropy is fitted by three exponentials and a constant term, the long relaxation time is simply obtained twice (Table 1). Moreover, measurements extended over a larger time window of 470 nsec demonstrated that up to 1 μsec no further relaxation process exists. These results indicate that the MalNPyr label attached to LPase undergoes two relaxation processes.

The MalNPyr label can also be attached to cysteine residues other than Cys-148. Such labels exhibit the same relaxation behavior as the one attached to Cys-148 (Table 1). Hence, all MalNPyr labels bound to LPase provide evidence for the existence of a slow relaxation process in the range of 50 nsec. In contrast, the product of the reaction of MalNPyr and mercaptoethanol (2-(2-hydroxyethylsulfuryl)-*N*-(1-pyrenyl)succinimide; MalNPyr-ME), when dissolved in a



membrane of [Myr_2]PtdCho, relaxes with ϕ = 5.3 nsec and does not exhibit a slow relaxation process (Table 1).

To investigate the putative correlation between internal dynamics and transport of LPase, we performed FAD measurements under conditions where transport is known to be blocked or at least reduced. One such case is precipitated LPase, which neither binds nor transports substrate (11). Fig. 1*C* shows that the MalNPyr label of precipitated LPase still exhibits the fast relaxation process but no longer exhibits the slow process. The latter is either absent or its relaxation time has become much longer, $\phi_2^{Pyr} > 500$ nsec. This ambiguity cannot be resolved because the anisotropy in Fig. 1*C* does not reach a constant value at long times, presumably due to rotational diffusion of small aggregates of inactive LPase.

A second possibility of reducing the transport activity of LPase is to decrease the temperature below the lipid phase transition (7–9). Fig. 2 shows the temperature dependence of the relaxation times and amplitudes of the tryptophan residues and the MalNPyr label in the range from 12°C to 36°C, which includes the lipid phase transition at T_t = 24°C. The two tryptophan relaxation times with their amplitudes as well as the fast MalNPyr relaxation time with its amplitude are either constant or vary smoothly; no drastic change is observed at T_t. By contrast, the slow relaxation time of the MalNPyr label increases considerably below T_t and becomes as large as 300 nsec at 13°C. Its amplitude decreases by a factor of two upon passing below T_t. Thus, the slow relaxation of the MalNPyr label becomes still slower and smaller in amplitude at $T < T_t$. This result suggests a correlation between internal dynamics and transport of LPase.

ANALYSIS AND DISCUSSION

Assignment of Relaxation Processes to Fluctuations. Relaxation processes of tryptophan residues in the picosecond and nanosecond time range up to 10 nsec have been attributed in other cases to orientational fluctuations of the indole group relative to the polypeptide backbone (10, 26). Hence, we assign the two tryptophan relaxations with 1 and 7 nsec to such fluctuations. Analogously, the 1- and 4-nsec relaxations of the two MalNPyr labels may reflect fluctuations of the MalNPyr group relative to the polypeptide backbone. This argument is supported by the fact that MalNPyr-ME dissolved freely in a lipid membrane relaxes with 5 nsec. In turn, the slow relaxation with 50 nsec exhibited by the tryptophan residues and the MalNPyr labels may be assigned to fluctuations of the polypeptide backbone. We note that the relaxation time for rotational diffusion of the entire LPase molecule in [Myr_2]PtdCho at 28°C has been determined experimentally as 25 μsec (5) and the relaxation time for wobbling of an LPase molecule about the membrane normal can be estimated in a way similar to the estimate given below for a membrane-spanning helix as 1 μsec. Thus, orientational fluctuations of the entire LPase molecule are much slower than the observed relaxation process with 50 nsec. According to the best available models for the structure of LPase (3, 6), the tryptophan residues and the cysteine residues bearing the MalNPyr labels are located on membrane-spanning helices. Therefore, we tentatively assign the slow relaxation of the

FIG. 1. Intensity (upper curves) and anisotropy (lower curves) of the tryptophan fluorescence of LPase reconstituted in [Myr_2]PtdCho (*A*), of the pyrene fluorescence of MalNPyr-Cys-148-labeled LPase reconstituted in [Myr_2]PtdCho (*B*), and of the pyrene fluorescence of MalNPyr-Cys-148-labeled LPase precipitated in inactive form (*C*). All measurements were done at 28°C. The experimental data are represented by dots, and the curves are least-square fits, with the goodness of the fits given by the residuals for the sum (RES_s) and difference (RES_d) of the parallel and perpendicular polarized fluorescence light.

Table 1. Relaxation times ϕ, relaxation amplitudes b, and residual anisotropies r_∞ of the tryptophan residues and MalNPyr labels of LPase reconstituted in [Myr_2]PtdCho and of MalNPyr-ME dissolved in [Myr_2]PtdCho at T = 28°C

	ϕ_1, nsec	b_1	ϕ_2, nsec	b_2	ϕ_3, nsec	b_3	r_∞	χ^2
Trp residues*	1.06	0.013	6.7	0.051			0.068	1.01
MalNPyr-Cys-148	4.2 ± 1.7	0.023 ± 0.005	38.6 ± 5.6	0.037 ± 0.003			0.128 ± 0.001	1.06
	4.2	0.023	37.4	0.020	40.1	0.016	0.128	1.06
MalNPyr-Cys-*X*	1.1	0.014	44.0	0.012			0.108	1.63
MalNPyr-ME	5.3	0.194					0.011	1.20

X, Position numbers of other cysteine residues.
*Analysis of the data of Fig. 1*A* within a time window of 12 nsec.

tryptophan residues and the MalNPyr labels to orientational fluctuations of membrane-spanning helices about their short axes.

Three further independent arguments may be given in support of this assignment. (*i*) There is the absence of a slow process in the relaxation of MalNPyr-ME in a lipid membrane. (*ii*) Polypeptides such as melittin and alamethicin may form membrane-spanning helices and as such exhibit a slow relaxation process with ≈40 nsec (17, 27). (*iii*) The relaxation time of a membrane-spanning helix may be estimated using Eq. 1. The experimental data for the amplitude of the slow relaxation process and for the limiting anisotropy of the MalNPyr label permit the determination, by means of Eq. 2, of the mean-square orientational fluctuations as $\langle\vartheta^2\rangle = 0.08$, equivalent to rms fluctuations of $\langle\vartheta^2\rangle^{1/2} = \pm 0.26$ or ± 15°. By using this value and the value $D_\perp = 5 \times 10^5 \cdot sec^{-1}$ derived for the relevant diffusion coefficient, one obtains ϕ = 60 nsec for the relaxation time of a membrane-spanning helix. This result is in good agreement with the experimental slow relaxation time.

Relationship Between Fluctuations and Function. To provide evidence for a putative coupling between internal dynamics and transport of LPase, two cases were investigated where transport was known to be blocked or at least reduced. (*i*) Precipitated LPase is completely inactive in the sense that it does no longer bind substrate. We found that precipitated LPase does not show the slow MalNPyr fluctuations. (*ii*) Below the lipid-phase transition the transport rate is decreased drastically (7–9), although binding is unaltered in consonance with unaltered secondary structure (6). Here, we found a reduced relaxation rate of the slow MalNPyr fluctuations together with a reduced amplitude. The faster relaxation processes of the tryptophan and MalNPyr labels were hardly affected by the lipid phase transition. These results suggest that a correlation between fluctuations and transport indeed exists and that this correlation is more pronounced the slower the fluctuations are.

This correlation may even be expressed in a quantitative manner. For this purpose, we use the Kramers model for chemical reactions. This model requires that the reaction involves a transition between two states separated by an energy barrier (Fig. 3). This condition is fulfilled in our case because the temperature dependence of the transport rate obeys an Arrhenius relation (9, 28). A transport cycle involves two conformational changes, one with bound substrate and one without. For LPase, however, the two rate constants are of similar magnitude (4); therefore we consider only one process with a rate equal to twice the transport rate. To this process we apply the Kramers relation adapted to the case of rotational diffusion. The prefactor k' in the Arrhenius

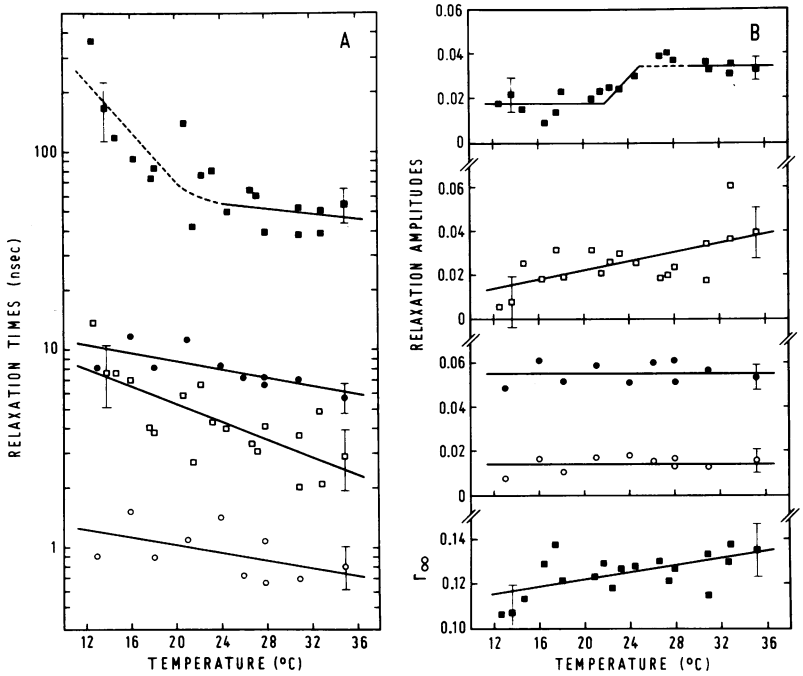


FIG. 2. Temperature dependencies of the relaxation times (*A*) and of the amplitudes as well as the residual anisotropy r_∞ (*B*) of the tryptophan residues (○, ●) and of the MalNPyr-Cys-148 label (□, ■) of LPase reconstituted in [Myr_2]PtdCho. In each case, open symbols denote the faster relaxation process, closed symbols the slower one. The parameters plotted were obtained from fits of experimental data such as those in Fig. 1 *A* and *B*. The residual anisotropy of the tryptophan residues could not be determined.

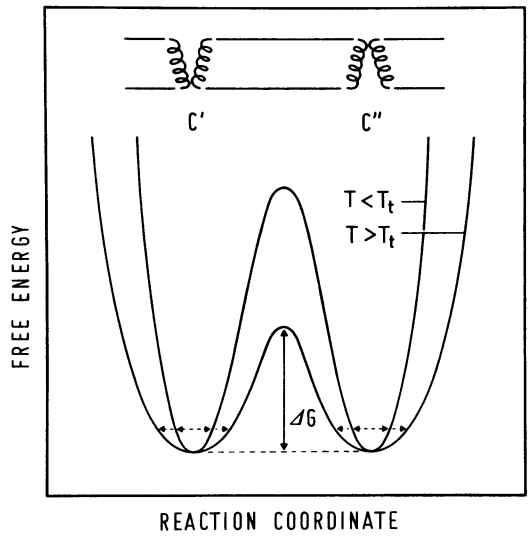


FIG. 3. Schematic plot of the free energy of LPase as a function of the reaction coordinate. The states C' and C'' represent the two conformations involved in transport, one with the binding open to one side of the membrane and closed to the other, and vice versa for the other conformation. The arrows indicate the amplitude of the thermal fluctuations in the two conformations. If the amplitude decreases at $T < T_t$, while the conformations remain unaltered, the energy barrier ΔG must increase.

relation is then given by Eq. 3, and insertion of $\phi = 50$ nsec, the relaxation time of the slow fluctuations expected to be most strongly correlated with transport, leads to $k' = 2 \times 10^6 \cdot \text{sec}^{-1}$. The experimental value for the transport rate k of LPase in fluid membranes is of the order of 10 sec^{-1} (4), so that the Arrhenius relation $k = k' \exp(-\Delta G/RT)$ requires an activation energy of $\Delta G = 30$ kJ/mol. Measurements of the temperature dependence of k in fluid membranes led to activation energies of 40–60 kJ/mol for cells (9) and 30–50 kJ/mol for reconstituted LPase (28), in rough agreement with the above value. Hence, the experimental values for k and ΔG are compatible with the assumption that the rate k' is determined by the slow orientational fluctuations seen. Thus, our data can be interpreted quantitatively in terms of a coupling between fluctuations and transport. This analysis, however, represents no proof for such a coupling.

Upon passing through the lipid phase transition the transport rate decreases by several orders of magnitude (9, 28). In our experiments we found that the relaxation time of the slow fluctuations increases to 300 nsec at 13°C so that k' decreases by a factor of 6 to $k' = 3 \times 10^5 \cdot \text{sec}^{-1}$. Hence, the low transport rate at $T < T_t$ must be caused mainly by a higher activation energy ΔG. Assuming $k = 10^{-3}$ sec^{-1} and the above value for k', the Arrhenius relation requires $\Delta G = 50$ kJ/mol—i.e., an increase by almost a factor of 2 compared with the value at $T > T_t$. Experimental results on ΔG at $T < T_t$ are not available. Our data, however, provide indirect evidence for a higher activation energy at $T < T_t$. The decrease of the relaxation amplitude of the slow fluctuations upon passing through T_t implies a decrease of the mean-square amplitude $\langle \vartheta^2 \rangle$ of these fluctuations. As indicated in Fig. 3, a smaller amplitude of the fluctuations in the two conformations requires a higher barrier between them. Thus, the Kramers model provides a description of the putative coupling between fluctuations and transport not only above but also below the phase transition.

In a final step of interpreting our data, we may come back to the picture of the slow fluctuations as representing orientational fluctuations of membrane-spanning helices. This picture fits perfectly with the model that transport involves a conformational change between two states exposing the binding site to one or the other side of the membrane. Because LPase is considered to be folded in membrane-spanning helices, this conformational change may be visualized as a change in the orientation of such helices (Fig. 3). In each of the two conformational states the helices would perform small orientational fluctuations with characteristic times of 50 nsec. By these helix fluctuations the protein would try to undergo the conformational change and would succeed about once in a million trials.

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