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Enhanced internal dynamics of a membrane transport protein during substrate translocation

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Abstract

Conformational changes are essential for the activity of many proteins. If, or how fast, internal fluctuations are related to slow conformational changes that mediate protein function is not understood. In this study, we measure internal fluctuations of the transport protein lactose permease in the presence and absence of substrate by tryptophan fluorescence spectroscopy. We demonstrate that nanosecond fluctuations of α-helices are enhanced when the enzyme transports substrate. This correlates with previously published kinetic data from transport measurements showing that millisecond conformational transitions of the substrate-loaded carrier are faster than those in the absence of substrate. These findings corroborate the hypothesis of the hierarchical model of protein dynamics that predicts that slow conformational transitions are based on fast, thermally activated internal motions.

Keywords: helix fluctuations; lactose permease; nanosecond motions; protein dynamics; time-resolved tryptophan fluorescence anisotropy

Protein motions perform most intricate tasks as is formidably exemplified by the action of transport proteins. Embedded in membranes, they catalyze the specific passage of substrate molecules from one side of the membrane to the other (Henderson, 1993; Kaback et al., 1994). They do so by switching between two main conformations exposing the substrate binding site alternatively to either side of the membrane. One essential and remarkable characteristic of these motions is that they occur in ways always ensuring a tight seal against other molecules and solutes—no matter if the transporter is empty or ligand-"filled." Although a wealth of kinetic data is available describing the rates of transport, we are far from understanding on a molecular-mechanical level this dynamic nature of transport proteins that lies at the heart of their biological activity. This is not only due to a deficit in structural information on the atomic level, but stems mainly from the difficulty to measure the internal protein motions that would then enable us to correlate them with the kinetic characteristics of protein functioning.

LP from the inner membrane of *Escherichia coli* is a paradigm for transport proteins (Kaback et al., 1994). It is a member of the family of 12-transmembrane helix transporters and mediates coupled transport of galactosides and protons across a membrane (Henderson, 1993; Kaback, 1997). Typical translocation rates k_{cat} of LP (Garcia et al., 1983; Wright, 1986) and of other transporters (Loo et al., 1998) are in the range of 10–100 s^{-1}; substrate binding can be considerably faster (Garcia et al., 1983; Wright, 1986; Wright et al., 1994). Spectroscopic and biochemical analysis of a series of single amino acid mutants indicates that there is considerable conformational flexibility in LP (Jung et al., 1994; Wu & Kaback, 1997; Weinglass & Kaback, 1999). Especially, helix motions that occur in the nanosecond time range were suggested to be linked to the much slower large-scale conformational rearrangements eventually leading to transport (Dornmair & Jähnig, 1989). To examine the validity of this hypothesis, we directly measured here these fast motions for liposome-reconstituted purified LP under various transport conditions. This could be achieved by utilizing LP's six tryptophan residues, which are all predicted to reside on membrane-spanning α-helices (Kaback et al., 1994), as intrinsic fluorescent probes for time-resolved fluorescence depolarization experiments. This approach allowed us to measure internal orientational fluctuations of not artificially labeled, fully functional LP both in the absence and presence of substrate, to our best knowledge the first measurement of this kind for a transport protein.

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Abbreviations: LP, lactose permease; TDG, thiodigalactoside.

Results and discussion

We studied orientational fluctuations of LP by time-resolved fluorescence depolarization experiments utilizing its six tryptophan residues as intrinsic fluorescent probes. This yields the time courses of the fluorescence intensity and anisotropy (Holzwarth, 1995) (see Materials and methods; Fig. 1A). The molecular orientational fluctuations are reflected by the anisotropy decay. The time window in which the anisotropy can be detected depends on the range of the intensity decay (also containing information about the excited electronic state of the fluorophores). Similar to other proteins, LP's tryptophan fluorescence intensity has—in addition to shorter lifetimes (Chen et al., 1991) ($\tau_1 - \tau_4$)—a small amplitude, long lifetime component (τ_5) of about 30 ns (Döring et al., 1995, 1997) (Table 1). This long lifetime enabled us to observe the anisotropy decay on a timescale of several tens of nanoseconds. In this regime, motions of side chains and secondary structural elements are expected to occur (Döring et al., 1997).

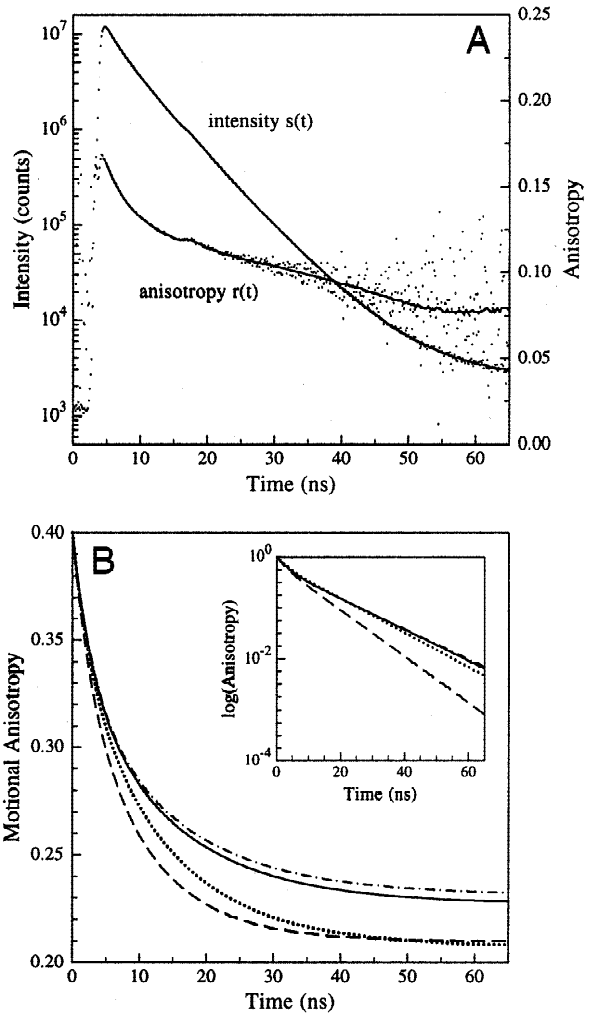

Fig. 1. A: Time course of the measured intensity and anisotropy of the tryptophan fluorescence of LP in the absence of substrate. The experimental data (dots) are presented together with fits (lines) of multi-exponential decays convoluted with the apparatus response function (the "shape" of the exciting light pulse). **B:** The relaxation times ϕ_i and amplitudes β_i of the anisotropy decays in the absence and presence of sugars as obtained from the fits (Table 1) are replotted as "*motional* anisotropy decays." $R_m(t) = \sum_{j=1-2}\beta_j \exp(t/\phi_j) + \beta_3$. In this form, the anisotropy decays reflect only molecular motions and are free from influences of the apparatus response function and certain spectroscopic properties of the tryptophan fluorescence on the measured anisotropy decay (Döring et al., 1995, 1997; see also Materials and methods). No substrate, ——; 200 mM lactose, – – –; 5 mM TDG, · · ·; 200 mM sucrose —·—. The dissociation constants for lactose and TDG are 15 mM and 70 μM, respectively, sucrose does not bind to LP. Inset: logarithmic *motional* anisotropy $\log[(R_m(t) - \beta_3)/(\beta_1 + \beta_2)]$.

Two types of internal fluctuations were indeed detected for LP as reflected by two anisotropy relaxation processes (Table 1; Fig. 1). External motions like rotational diffusion of the entire protein within the viscous membrane or tumbling of the membrane vesicle are too slow to be detectable in a nanosecond time window (Dornmair et al., 1985) making membrane proteins ideal candidates for the study of internal nanosecond fluctuations. As established in previous studies (Beecham & Brand, 1985; Döring et al., 1995, 1997), the faster of the two measured relaxation processes is caused by wobbling motions of the tryptophan side chains relative to the backbone and the slower relaxation process by protein domains, which in the present case may be either fluctuations of individual α-helices or groups of α-helices (Dornmair & Jähnig, 1989).

We investigated how these internal fluctuations of a transport protein are affected by substrate transport. Adding transportable galactosides to LP at saturating levels resulted in faster decays of the anisotropy indicating enhanced fluctuations. This substrate ef-

Table 1. *Parameters from the fits to the decays of the tryptophan intensity and anisotropy of LP: intensity lifetimes τ_i, intensity amplitudes α_i, anisotropy relaxation times ϕ_i, anisotropy amplitudes β_i, and angles between excitation and emission dipoles $\theta_{e,i}$ (see Materials and methods)*[a]

		No substrate	Lactose	TDG	Sucrose
τ_1	(ns)	0.1	0.4	0.1	0.6
τ_2		1.1	1.4	1.7	2.6
τ_3		3.6	3.3	4.1	4.8
τ_4		6.0	5.8	6.4	6.4
τ_5		33	19	33	33
α_1		0.36	0.19	0.50	0.20
α_2		0.13	0.15	0.13	0.20
α_3		0.25	0.32	0.22	0.39
α_4		0.27	0.33	0.14	0.22
α_5		0.0005	0.0012	0.0005	0.0010
ϕ_1	**(ns)**	**3.0**	**2.6**	**2.5**	**3.1**
ϕ_2		**14.2**	**9.6**	**12.9**	**14.7**
β_1		**0.066**	**0.053**	**0.053**	**0.070**
β_2		**0.11**	**0.14**	**0.14**	**0.10**
β_3		**0.23**	**0.21**	**0.21**	**0.23**
θ_{e1}	**(deg)**	**38**	**38**	**37**	**38**
θ_{e2}		**47**	**49**	**46**	**48**

[a] The anisotropy relaxation times ϕ_i and the anisotropy amplitudes β_i (in bold) reflect internal orientational fluctuations of LP and are replotted as motional anisotropy decays R_m in Figure 1B. The substrate concentrations are 200 mM lactose and 5 mM TDG, as a control 200 mM sucrose was used (dissociation constants for lactose and TDG are 15 mM and 70 μM, respectively; sucrose does not bind to LP). The standard deviations are 0.1 ns for τ_1–τ_4, 2 ns for τ_5, 0.01 for α_1–α_4, 0.0002 for α_5, 0.1 ns for ϕ_1, 0.5 ns for ϕ_2, 0.01 for β_1 and β_2, and 0.5° for θ_{e1} and θ_{e2}.

fect on the relaxation times ϕ_i and amplitudes β_i of the anisotropy decays is illustrated in Figure 1B. To extract the characteristics of these underlying molecular wobbling motions, we converted (van der Meer et al., 1984) these relaxation times ϕ_i and amplitudes β_i to rotational diffusion constants $D_{\perp i}$ and root-mean-square (RMS) amplitudes $\vartheta_{\mathrm{RMS},i}$ (Fig. 2). We found that substrate addition mainly affects LP's α-helix fluctuations (relaxation process 2) while the fluctuations of the tryptophan side chains themselves (relaxation process 1) are hardly changed (Fig. 2). The diffusion coefficient $D_{\perp 2}$ of the helix fluctuations is increased by 80% upon lactose addition (from 3.8 to 6.9 μs^{-1}) and by 40% upon addition of the artificial substrate thiodigalactoside (TDG) (to 5.1 μs^{-1}); hence, the fluctuations become faster. The effect on the RMS amplitudes $\vartheta_{\mathrm{RMS},2}$ of these orientational motions is less dramatic: they increase by 10% (from 20 to 22°) indicating a slight increase of the angle covered by the fluctuating helices. The extent of these substrate effects is remarkable because the detected motions are an average measured over all tryptophans present in LP. In summary, α-helix dynamics are increased globally in the transport protein when it translocates substrate across the membrane. Adding sucrose, which is not bound by LP, does not change the internal fluctuations significantly.

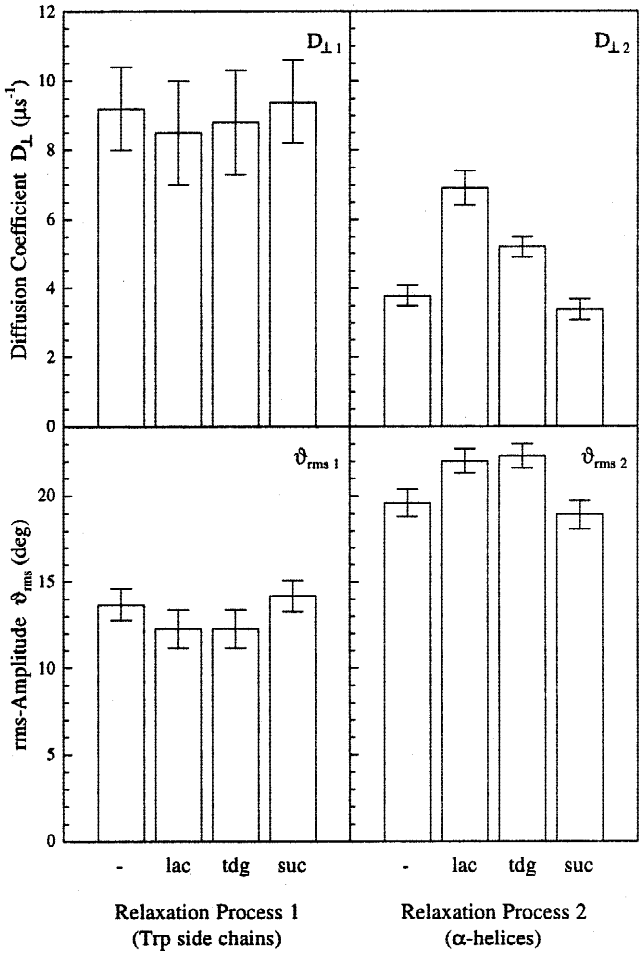

Fig. 2. Diffusion coefficients $D_{\perp,1}$ and $D_{\perp,2}$ and RMS amplitudes $\vartheta_{\mathrm{RMS},1}$ and $\vartheta_{\mathrm{RMS},2}$ of the two measured relaxation processes of LP. These relaxation processes 1 and 2 are interpreted to arise from tryptophan side-chain fluctuations and α-helix fluctuations, respectively.

We can now ask whether there is a correlation between nanosecond internal motions and biochemically measured millisecond conformational changes. Kinetic analysis of transport measurements reveals that substrate-loaded LP performs these millisecond conformational transitions 5–30 times faster than empty LP in the absence of a membrane potential (Garcia et al., 1983; Wright, 1986; Kiefer, 1992) (Fig. 3); a similar behavior was found for other 12-transmembrane helix transporters (Wright et al., 1994). Additionally, LP translocates lactose two to six times faster than the artificial substrate TDG (Garcia et al., 1983; Wright, 1986; Kiefer, 1992). This can be compared with the nanosecond fluctuations of LP's α-helices measured in this paper. They are slowest for "empty" LP, faster for TDG-loaded LP, and fastest during lactose translocation. While the timescales of these two types of protein motions are about six orders of magnitude apart, substrates affect these motions in the same order on both timescales. This remarkable correlation suggests that conformational changes leading to substrate translocation are causally related to the much faster nanosecond fluctuations.

This result agrees well with the concept of protein energy landscapes that has been developed on the basis of low temperature studies of myoglobin (Ansari et al., 1985). Briefly, it states that folded proteins can adopt a large number of conformational substates with slightly different potential energies (Frauenfelder et al.,

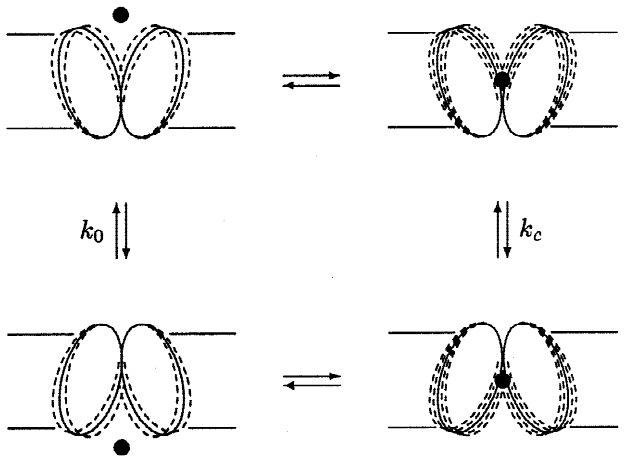

Fig. 3. Minimal kinetic model for the transport cycle of LP (adopted from Patlak, 1957; LP's two substrates, one sugar molecule and one proton, are represented as a single filled black circle). The transport cycle consists of four steps: substrate binding, a major conformational transition (with rate k_c) exposing the substrate to the other side of the membrane, substrate dissociation, and finally a second major conformational change (with rate k_0) reorienting the binding site back to the initial side of the membrane. Substrate binding and dissociation are fast as compared to the two conformational transitions (Garcia et al., 1983; Wright, 1986). The slowest step in this cycle is transposition of the empty binding site. In the absence of a membrane potential, k_0 is about 5–10 times slower than k_c for TDG and about 15–30 times slower than k_c for lactose (Garcia et al., 1983; Wright, 1986). A recent reinvestigation of these rates for LP reconstituted in lipid vesicles (under conditions very similar to our fluorescence anisotropy measurements) agrees well with these ratios, the values obtained at pH 7.3 were $k_0 = 1.6\ s^{-1}$, $k_{c,\mathrm{TDG}} = 6.0\ s^{-1}$, and $k_{c,lactose} = 23.8\ s^{-1}$ (Kiefer, 1992). The fluorescence measurements of the present study were either performed in the total absence of substrate or in the presence of equal substrate concentrations on both sides of the membrane. Under these conditions, LP performed conformational transitions exclusively either with the rate k_0 or k_c, respectively.

1997). A hierarchy of internal fluctuations covering a broad range of timescales corresponds to transitions between all these substates. To which extent which fast internal fluctuations are coupled to major conformational changes involved in protein function on slower timescales has since then been an open question (Frauenfelder & Leeson, 1998). We have presented here one of the rare studies addressing this question by directly measuring fast internal protein dynamics under close-to-native conditions of protein functioning: galactoside transport at ambient temperatures.

We conclude that substrate binding appears to alter the energy landscape of LP in a way that helix fluctuations can sample the accessible conformational space more efficiently. On the level of helix fluctuations, the protein becomes *more dynamic*. The fluctuations of the tryptophan side chains themselves are hardly altered (see also Ansari et al., 1985), most probably because the tryptophans are not close to the binding site (Weitzman et al., 1995). On the other hand, a galactoside binding protein behaves completely different from a galactoside transport protein. For maltose-binding protein, we observed the opposite dynamic behavior in response to substrate binding (Döring et al., 1999). When it closes its binding cleft around the ligand, its domain motions are strongly restricted. Hence, on the level of secondary structure elements or protein domains the protein becomes *less dynamic* upon ligand binding. Since the energy landscape determines the internal protein dynamics, it is this energy landscape that appears to be specifically designed for distinct protein functions.

The observed correlation between helix fluctuations and slow conformational changes might therefore be a consequence of the typical character of the energy landscape of a transport protein. Although conformational changes are often described by models implying an energy barrier along a low-dimensional reaction coordinate, similar to a chemical reaction (Kramers, 1940; Patlak, 1957), in the high dimensionality of phase space, a protein might be able to circumvent high energy barriers on a long journey through a labyrinth of pathways. Extensive sampling of the conformational space by thermally activated fluctuations will eventually lead to the passage through these paths corresponding to the large conformational change. The overall rate of the slow conformational transition would then depend strongly on how efficiently the diffusive motions explore the labyrinth. Such an interpretation of protein functioning is supported by our finding that fast internal helix fluctuations and slow conformational transitions in lactose permease are coupled. To which extent energy barriers have still to be overcome and therefore contribute to the rate of conformational changes is an intriguing question for future studies.

Materials and methods

Purification and reconstitution

LP was purified from strain T206 and reconstituted in lipid vesicles essentially as described (Dornmair, 1988). However, an additional purification step using S Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) in 20 mM MES, 0.1 mM Na-EDTA, 1 mM DTT, 0.01% dodecyl maltoside, pH 6.2 was performed, followed by adjusting the dodecyl maltoside concentration to 0.1% and reconstituting the protein in vesicles of a mixture of 1-palmitoyl-2-oleoylphosphatidyl-ethanolamine and 1-palmitoyl-2-oleoylphosphatidyl-glycerol at a molar ratio of 4/1 as described (Dornmair & Jähnig, 1989). The lipid/protein molar ratio was 1,000. The lipid concentration was determined using trace amounts of radioactively labeled lipid, and LP activity was measured in a binding assay. Mock reconstitutions for fluorescence background measurements were performed in the same way, however, in the absence of protein.

Time resolved fluorescence anisotropy (for a review see, for example, Holzwarth, 1995)

The fluorescence measurements were performed at room temperature as described (Döring et al., 1995, 1997). Tryptophan fluorophores of liposome-reconstituted LP are repeatedly excited with polarized picosecond light pulses. As light source, a mode-locked Nd-YAG-laser and a cavity-dumped dye laser were used with a pulse repetition rate of 4.1 MHz. The excitation wavelength was 300 nm. Each pulse selectively excites a population of fluorophores with their absorption dipole moments mainly parallel to the polarization of the exciting light. The emitted light is detected at 350 nm under two orthogonal polarizations—one parallel and the other perpendicular to the polarization of the excitation light. The time courses of these two fluorescence intensity decays are monitored by time-correlated single photon counting (Holzwarth, 1995). The total intensity is $s(t) = i_{\parallel}(t) + 2Gi_{\perp}(t)$ and the anisotropy is defined as $r(t) = [i_{\parallel}(t) - Gi_{\perp}(t)]/s(t)$, with G representing a scaling factor determined experimentally by calibration.

Data analysis

The experimental data were analyzed as described (Döring et al., 1997). Briefly, a multi-exponential decay $S(t) = \sum_{i=1-5}\alpha_i \exp(-t/\tau_i)$ was assumed for the total intensity. For the anisotropy, a modified multi-exponential decay $R(t) = f(t)\{\sum_{j=1-2}\beta_j \exp(t/\phi_j) + \beta_3\}$ with $f(t) = \{\sum_{i=1-4}P_2(\cos\theta_{e1})\alpha_i \exp(-t/\tau_i) + P_2(\cos\theta_{e2})\alpha_5 \exp(-t/\tau_5)\}/S(t)$ was used to account for the more complex spectroscopy caused by a long lifetime component of tryptophan (Döring et al., 1995). The angles $\theta_{e,i}$ between the absorption and emission dipole moments had to be explicitly taken into account, since the longest lifetime component τ_5 originates from a different dipole moment. From these equations, expressions for the parallel and perpendicular components of the fluorescence intensity were derived and convoluted with the apparatus response function (determined by use of a strictly mono-exponential fluorescence standard) and fitted to the measured intensities $i_{\parallel}(t)$ and $i_{\perp}(t)$. The obtained parameters are presented in Table 1. The intensity data $i_{\parallel}(t)$ and $i_{\perp}(t)$ were background-corrected by subtracting mock-sample data from the actual protein sample data (Döring et al., 1997). To achieve a sufficient signal-to-noise ratio even at 50 ns after the excitation pulse, extensive data sampling over 10 h was required, demanding an extremely high light-source and overall-setup performance.

Molecular motions

Depolarization, as seen in the anisotropy decay, is caused by rotational motions of the excited fluorophores. The orientational fluctuations in a protein may be described as (angularly restricted) diffusional motions. Their velocity and amplitude can be expressed by rotational diffusion coefficients $D_{\perp}$ and RMS amplitudes ϑ_{RMS}. Their values can be calculated from the anisotropy decay: From the amplitudes β_j of the anisotropy decay, the orientational order parameters $\langle P_2\rangle_i$ can be derived according to $\langle P_2\rangle_1^2 = (\beta_2 + \beta_3)/(\beta_1 + \beta_2 + \beta_3)$ and $\langle P_2\rangle_2^2 = \beta_3/(\beta_2 + \beta_3)$. Since $\langle P_2\rangle_i =$

$\langle(3\cos^2\vartheta_i - 1)/2\rangle$ with ϑ_i denoting the angle of the fluorophore axis relative to the mean axis resulting from relaxation process i, in the limit of high order the RMS amplitude of ϑ_i is obtained as $\vartheta_{\mathrm{RMS},i} = [(2/3)(1 - \langle P_2\rangle_i)]^{1/2}$. The diffusion coefficient is given (van der Meer et al., 1984) by $D_{\perp,i} = (1 - \langle P_2\rangle_i)/6\phi_i$. Flexible molecules display a rapid anisotropy decay, i.e., short anisotropy relaxation times and/or high amplitudes for the internal relaxation processes, indicative of large rotational diffusion coefficients and large RMS amplitudes. Because the external rotational mobility of membrane proteins is hindered and too slow to be detected in a fluorescence depolarization experiment, they show a finite residual anisotropy (i.e., an additional amplitude in the anisotropy decay).

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