Functional Subconformations in Protein Folding: Evidence from Single-Channel Experiments

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We study fluctuations in ion conductance and enzymatic rates of the sugar-specific channel-forming membrane protein, Maltoporin, at the single-molecule level. Specifically, we analyze time-persistent deviations in the transport parameters of individual channels from the multichannel averages and discuss our findings in the context of static disorder in protein folding. We show that the disorder responsible for variations in ion conductance does not affect sugar binding, suggesting that Maltoporin can exist in a wide set of fully functional, yet distinctly different, subconformations.

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By analyzing fluctuations in biochemical processes, it is possible to reveal important kinetic features that are usually hidden in average rates. This is particularly true for a variety of modern techniques that can probe the functioning of proteins on a single-molecule level. Among the new questions to address is that of static and dynamic disorder in protein folding. In experiments dealing with single protein molecules, static disorder is seen as time-persistent deviations of individual reaction rates from their ensemble average. Dynamic disorder can often be observed as rate variations with time. This new knowledge advances our understanding of protein folding and misfolding in living cells—a subject of both theoretical and practical significance [1].

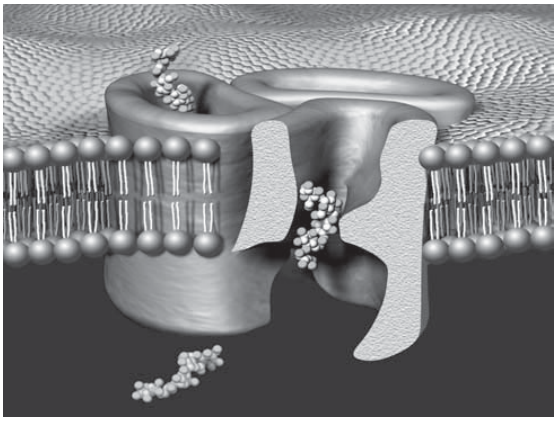
Reconstitution of ion channels into planar lipid bilayers is the oldest technique that allows interrogation of single molecules in their functional states [2,3]. The spontaneous insertion of a single channel is detected as a rapid stepwise increase in the ion current through the membrane. From the first reconstitution experiments [2,3] it became clear that the ''discrete conductance steps'' show certain variation around their average value and that this variation is significantly larger than the accuracy of the measurement. Nevertheless, with one remarkable exception [4], for more than 30 years of experimentation the variability of conductance from channel to channel has never managed to attract any in-depth discussion of its origin. Among the possible reasons for such neglect could be the following two: (i) most of the researcher's attention was concentrated on the average conductance, and (ii) a proper language to discuss this functional variability was not available.

In the present study we compare functional properties—ion conductance and sugar binding—of 130 single Maltoporin channels reconstituted into lipid bilayers, and interpret our findings using the language of static disorder in protein folding [5,6]. We focus on time-persistent deviations in parameters of individual channels from their ''ensemble'' averages. The stretched-exponential or $1/f$ behavior of conductance fluctuations of the Maltoporin channel in time was investigated previously [7,8] and attributed to conformational fluctuations related to protein dynamic disorder [9]. An alternative explanation could be based on the idea of multiple coupled sites for ligand binding [10]; however, in the case of ions inside the channel the characteristic time scale seems to be too fast.

Maltoporin (also called LamB) is a solute-specific channel that facilitates transport of maltose-based sugars across bacterial membranes [11,12]. Figure 1 (top) shows a cartoon of the Maltoporin channel in the presence of permeating sugars. According to x-ray crystallography [13], the Maltoporin channel is a trimer, wherein each monomer is a water-filled β barrel with short turns at the inner side and large irregular loops at the outer side of the cell. The third loop folds inside the β barrel and forms a constriction at the middle of the pore, giving it an hourglass shape. The structure of sugar-soaked crystals of Maltoporin shows a specific sugar translocation pathway [14].

Maltoporin channels have been extensively studied using planar lipid bilayers, a technique which offers the advantages of a well-controlled environment. When reconstituted into a membrane, Maltoporin forms ion-permeable channels [15]. Penetrating sugars bind to Maltoporin and simultaneously reduce the small-ion permeation [16]. Recently we investigated the ''enzymatic activity'' of Maltoporin—the ability to bind and facilitate maltosugar transport—at the single-molecule level [17]. We have shown that: (i) single-molecule events of sugar translocation can be observed as transient interruptions in the ion current through the Maltoporin channel; (ii) one sugar molecule completely blocks ion current through one of the pores of the trimer; (iii) monomers bind sugars independently of each other. Thus, we can study channel enzymatic activity concurrently with its ability to conduct small ions, e.g., potassium and chloride.

Sugar molecules diffuse through the channel pores interacting with their interior via van der Waals forces and hydrogen bonding. The constructive role of these interactions was recently elucidated in a theoretical model



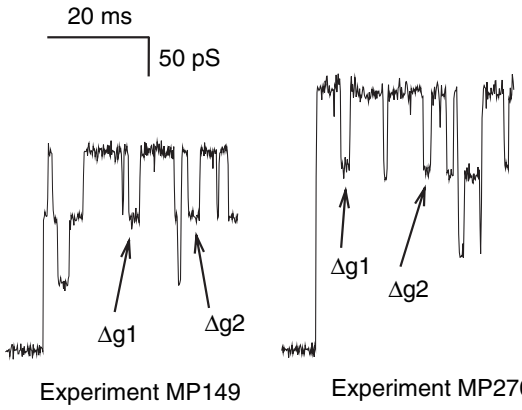


FIG. 1. (Top) A sketch of a trimeric Maltoporin channel reconstituted into a lipid bilayer in the presence of penetrating sugar molecules. Each subunit is about 80 Å long. The natural function of Maltoporin is to mediate passive sugar translocation. (Bottom) Judging by conductance, channels occur in different sizes. Transient blockages of ion currents through individual monomers by sugar molecules allow us to probe conductances of all three monomers comprising one channel. $\Delta g1$ and $\Delta g2$ denote the pairwise consecutive measurements of monomer conductances from the same Maltoporin trimer (see also Fig. 3). The diphytanoylphosphatidylcholine bilayer membrane was bathed by 1 M KCl, 1 mM $CaCl_2$, and 10 mM Tris buffered to *p*H 7.4. Transmembrane voltage was 200 mV unless otherwise stated; temperature was $23 \pm 0.5\,^{\circ}$C. Other technical details can be found elsewhere [7,17].

for single particle diffusion in the potential of mean force [18].

Figure 1 (bottom) contains ion current recordings of two different single Maltoporin channels in the presence of 10 μM sugar (maltohexaose) in the membrane-bathing KCl solution. Penetrating sugar molecules transiently interrupt potassium and chloride ion currents, inducing blockages by one and two thirds of the initial channel conductance.

Importantly, all 130 channels of the present study, including these two, were reconstituted, one at a time, using identical experimental protocol and conditions (see Fig. 1 caption); however, it is clearly seen that the channels differ by their conductance. Conductances of the trimers (fully open, unblocked channels, Fig. 1, bottom) ranged between 230 and 350 pS. Their distribution was well described by a single Gaussian centered at 298 pS with the half-height width of 39 pS (corresponding to the standard deviation of 17 pS).

The current recordings also demonstrate that the dwell time of a sugar molecule in the channel is of the order of 10^{-3}–10^{-4} s. It is interesting to compare this time with the characteristic time of diffusional relaxation expected for a molecule of this size in a channel of 5 nm length. For a cylindrical channel of length L and radius a, the average lifetime of a particle that is initially uniformly distributed in the channel is given by [19]:

$$\tau = \frac{L^2}{12D}\left(1 + \frac{3\pi a}{2L}\right), \tag{1}$$

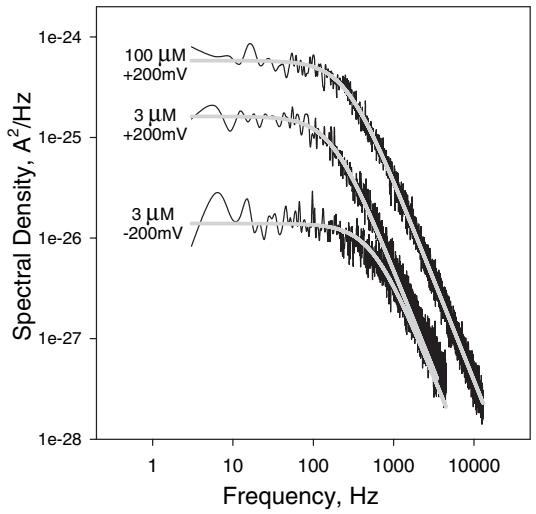
where it is assumed that particle diffusion in the channel and in the bulk is described by the same diffusion coefficient D. This expression accounts for the finite probability of particle return to the channel at finite L/a ratios. For the case of a long and narrow channel, where $L \gg a$, Eq. (1) is reduced to a well-known estimate $L^2/12D$ [20]. Substituting $L \approx 5$ nm and $D \approx 5 \times 10^{-10}$ m^2 s^{-1}, we obtain $\tau \approx 5 \times 10^{-9}$ s. In the absence of attractive interactions between the particle and the pore, diffusion within the pore can be slowed down by an order of magnitude [21], but even then the calculated time is about 4 orders of magnitude smaller than the time observed in the experiment. The long residence time is a result of strong interactions between permeating sugar molecules and the sugar binding regions within Maltoporin pores [14]. It is worth noting here that a similar significant increase in the particle residence time in the channel can also be found in the case of nonspecific interactions, e.g., for polyethyleneglycols in the alpha-Hemolysin channel [22].

Noise analysis of ion current through the trimer in the presence of sugar [7,17] shows that the power spectra (Fig. 2, top) can be well described by single Lorentzians with corner frequencies, f_c, that depend on the applied transmembrane voltage and sugar concentration. The main parameters of the enzymatic reaction are the on- and off-rates which can be found as:

$$k_{\mathrm{on}} = \frac{2\pi f_c p}{[c]}, \qquad k_{\mathrm{off}} = 2\pi f_c (1 - p), \tag{2}$$

where $[c]$ is sugar concentration, and p is the probability to find a monomer of the channel in the blocked state, measured as the relative average current decrease due to sugar addition. The binding constant is the ratio $k_{\mathrm{on}}/k_{\mathrm{off}}$.

First, to determine whether the fluctuations in ion and sugar transport parameters are correlated, we plot the sugar binding constant versus the trimer conductance. The data in Fig. 2 (bottom) show that deviations in the sugar binding constant and small-ion conductance from their average values are poorly correlated. Applying linear correlation



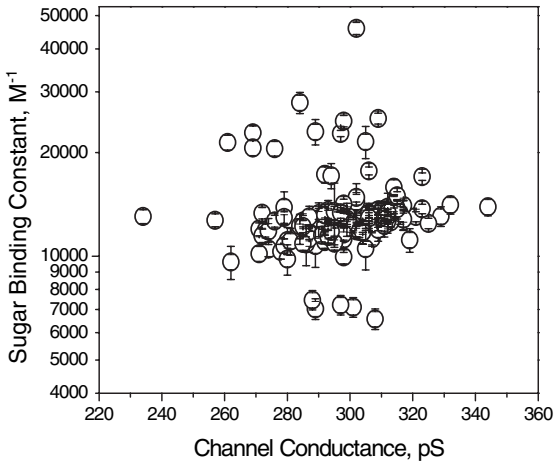


FIG. 2. (Top) Power spectra of ion current noise for two sugar concentrations and two applied voltages obtained from the same Maltoporin channel. Gray solid lines are Lorentzians fitted to the data. (Bottom) The static disorder in sugar binding and channel conductance show no correlation. The vertical error bars represent the standard deviation of the binding constant measurements; the error of the conductance measurement (horizontal bars) is approximately the size of the symbols.

analysis, we find that the correlation coefficient between the small-ion conductance and logarithm of the sugar binding constant is smaller than 0.05 with nearly zero regression slope. Similar results were obtained for the on- and off-rate constants (data not shown).

The absence of any significant correlations between the two parameters leads us to conclude that the static folding disorder, responsible for the time-persistent deviations of the channel conductance from its ensemble average, does not perturb the channel's ability to bind sugars. The channel is enzymatically functional in many different subconformations. Several research groups reached similar conclusions for the turnover rates of enzymes studied in single-molecule fluorescence experiments [23–26].

Second, we detect a significant degree of folding cooperativity among the monomers within a given trimer. Conductances of the pores within a trimer tend to be equal to each other. That is, our initial observation was that if the conductance of the trimer was higher than the average, then each of the three monomers had also higher conductance. And vice versa, if the trimer conductance was smaller than the average, then each of the three monomers had smaller conductance. To quantify this observation, we performed random sampling of conductances of the monomers comprising a particular trimer by measuring amplitudes of two time-resolved consecutive blockages of the trimer by sugar. Figure 1 (bottom) illustrates this procedure.

Figure 3 shows significant pairwise correlations in the consecutive measurements of monomer conductances, $\Delta g1$ and $\Delta g2$, within the same trimer. Linear correlation

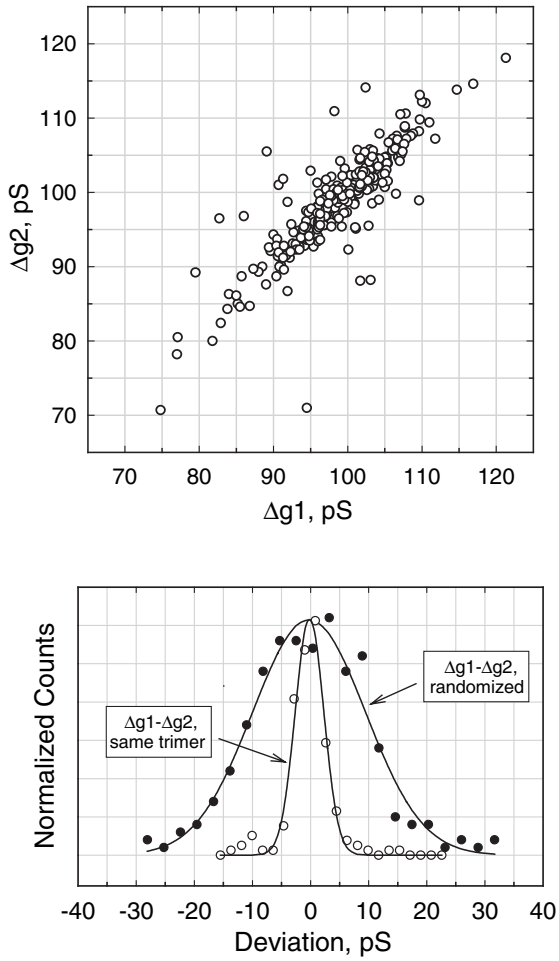


FIG. 3. Monomer conductances are more similar to each other within the same Maltoporin trimer than in the whole ensemble of measurements. (Top) Correlation between the conductance steps of the consecutive time-resolved blockages illustrated in Fig. 1. Each point on the graph represents a $\Delta g1$, $\Delta g2$ pair for an individual channel. (Bottom) Open symbols: a histogram of differences between $\Delta g1$ and $\Delta g2$ readings obtained from the same channels. Closed symbols: a histogram of differences for the randomized Δg readings. Counts are normalized to allow comparison of the histograms.

analysis of the data in Fig. 3 (top) gives the correlation coefficient of 0.87 with the regression slope close to unity. Figure 3 (bottom) demonstrates that deviations between $\Delta g1$ and $\Delta g2$ within an individual trimer are about fourfold smaller than deviations within the whole set of trimers. Thus, we find that in most cases the sugar-induced current steps reveal approximately equal ion conductances of the monomers comprising a trimer. Our tentative explanation is that monomers in the trimer communicate by adjusting their subconformations to each other, or, alternatively, by selecting each other at the oligomerization stage in the bacterial cells.

We interpret our findings using the concepts of protein folding disorder [5,6] according to which the native state of a protein is represented by a set of (slightly) different yet stable conformations. To help the skeptical reader, we offer a list of alternative explanations: (i) inhomogeneity in membrane lateral organization, which modifies channel properties locally [27], (ii) strongly bound components of bacterial membrane, i.e., lipopolysaccharides, which cause variability in channel properties, (iii) different amount of detergent (used to keep Maltoporin in a functional state in water solutions) trapped into the trimeric channel structure. The discrimination between these possibilities will require additional experiments with new methods. We note, however, that similar alternatives (with ''membrane'' substituted by ''immobilizing surface'', etc.) apply to all single-molecule studies reported so far.

One unsolved problem is to understand what particular subconformations could result in such features. Crystal structures are of little help primarily because the diffraction patterns of x-ray crystallography result from ensembles of scattering molecules. Heterogeneity of subconformations is difficult to identify and to model [29], so that averaged conformations are used instead. In the case of Maltoporin, the β barrel itself is recognized to be very rigid; therefore, it is an unlikely candidate for the locus of variability. It seems that the short turns at the periplasmic side do not have enough freedom, either. However, the large irregular loops folded on the extracellular side and, partially, inside the channel pore [13,14] could introduce a significant degree of static disorder and, therefore, contribute to persistent fluctuations in both the small-ion conductance and the sugar translocation studied here.

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