Transient Protein Softening during the Working Cycle of a Molecular Machine

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Proper functioning of proteins usually requires a certain internal flexibility provided by stochastic structural fluctuations on the picosecond time scale. In contrast with conventional steady-state experiments, we report on a novel type of (laser-neutron) pump-probe experiment combining *in situ* activation of protein function with a time-dependent test of protein dynamics using quasielastic neutron scattering. A ''transient protein softening'' is shown to occur during the photocycle of bacteriorhodopsin as a direct proof for the functional significance of protein flexibility.

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Proteins like enzymes, receptors or ion pumps can be viewed as ''molecular machines'', whose three-dimensional structures are well-adapted to their biological functions. However, protein functionality is often also influenced by or even restricted to certain environmental conditions such as temperature or hydration. Examples include proton translocation in bacteriorhodopsin [1], ligand binding to myoglobin (see, e.g., [2]), and photosynthetic electron transfer in plant photosystem II [3]. In these cases, the temperature-dependent efficiencies of functional processes are found to be correlated with the so-called ''dynamical transition'' [4] of the respective proteins. This transition represents the onset of localized diffusive protein motions on the picosecond time scale at temperatures above 200 K, i. e. the onset of internal stochastic structural fluctuations such as diffusive reorientations of methyl groups or other small molecular subgroups [5–7]. The dynamical transition and its dependence on external factors like protein hydration [8–10], lipid/protein ratio of the membrane [11], or solvent viscosity [12,13] has been studied extensively using quasielastic neutron scattering (QENS). This technique makes use of the large incoherent scattering cross section of protons and of their quasiuniform distribution in biomolecules, so that hydrogen motions image protein dynamics very well [14]. So far, however, dynamics-function correlations have only been studied indirectly in steady-state experiments by variation of the above-mentioned external parameters, while a direct experimental evidence for a time-dependent modulation of protein dynamics during functional operation is still lacking.

One prototypical model system for studying the structure-dynamics-function relationship is the light-driven proton pump bacteriorhodopsin (BR) embedded in the purple membrane (PM) of *Halobacterium salinarum*. BR is activated by absorption of a light quantum, which initiates a photocycle of well-defined protein-chromophore conformations (intermediates) characterized by specific absorption maxima and decay times ranging from 500 fs to about 15 ms in aqueous solution [1]. The structural changes accompanying the proton transport (see Fig. 1), are most pronounced in the *M* intermediate [15,16]. It is widely accepted that these structural modifications are a prerequisite for the vectorial character of the proton translocation. As an indication for a strong dynamics-function correlation, steady-state QENS experiments in the dark

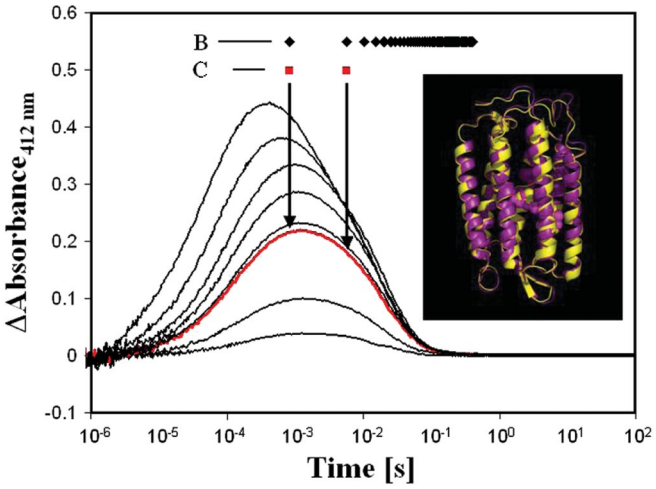

FIG. 1 (color). Absorbance changes of BR at 412 nm at room temperature as a function of time after laser excitation at 532 nm and $t = 0$. The actinic laser pulse energies are—from top to bottom—70, 36, 26, 18, 10, 8, 3.3, and 1.4 mJ/cm^2. Data were obtained directly at PM samples used for light-excited QENS experiments. Diamonds and squares indicate the arrival times of the neutron pulses at the sample relative to the laser pulse in two different measurement modes, i.e., experiments without (B) and with time selection (C), respectively. Here, the letters B and C correspond to the subscripts of the fit functions to the respective QENS spectra shown in Fig. 2. The inset shows the superposition of the structures of a BR monomer in the ground state BR_{568} (purple) and in the *M* intermediate (yellow), respectively, according to Sass *et al.* [16].

(see, e.g., [8,9]) have established that the picosecond diffusive protein motions are suppressed below a temperature of 230 K and at hydration levels lower than 70% relative humidity (r.h.) along with a cessation of the proton transport (see, e.g., [1,9]). In the present study, a modulation of protein dynamics during a functional process in BR is directly observed in a novel type of (light-neutron) pump-probe experiment, which combines *in situ* optical activation of the biological function of BR with a time-dependent test of its protein dynamics using QENS. This is achieved by synchronizing repetitive laser illumination of the sample with a pulsed QENS measurement. In this approach, QENS spectra resulting from individual neutron probe pulses reflect the protein dynamics of specific intermediate states provided that suitable delays between laser excitation and neutron probe pulses are chosen (see Fig. 1).

Experiments were performed using multilamellar stacks of aligned wild-type PM hydrated at 98% r.h. as described previously [9]. H_2O was substituted by D_2O to suppress the scattering contribution of the solvent. In PM about 75% of the total incoherent scattering is due to BR [9], so that the QENS results are dominated by the protein contribution. The *pD* value of the sample was adjusted to 6.5 using 50 mM imidazole to provide a sufficiently large pool of protons for the whole duration (6 h) of the repetitive laser-excited QENS measurements. PM samples were contained in vacuum-tight sample cells with sapphire windows, which are transparent to both, visible excitation light and neutron probe pulses, respectively.

For the special conditions of D_2O-hydrated PM films, the kinetics of the *M* intermediate of BR have been carefully studied by time-resolved optical absorption difference spectroscopy prior to the actual QENS experiment. A pulsed NdYAG-laser (Spectra Physics PRO-350) is employed as the actinic light source providing quasimonochromatic light pulses of ~6 ns duration at 532 nm, i.e., close to the peak absorption of BR_{568}. The induced absorption changes were monitored at 412 nm, which is the peak absorption wavelength of the *M* intermediate M_{412}. Figure 1 shows the laser-induced absorption changes as a function of laser pulse intensity. These data reveal that the time constants for formation and decay of *M* are virtually independent of actinic laser intensity below a threshold value of ~10 mJ/cm^2 per pulse. Above this value, a strongly nonlinear behavior with faster kinetics is observed indicating the presence of a branched photocycle, most likely due to the formation of the bleached intermediates *P* and *Q* absorbing close to the test wavelength of 412 nm [17]. The formation of *P* and *Q* has been associated with an irreversible bleaching of BR [17] observed before upon pulsed laser excitation at 532 nm [18]. In addition, the photocycle kinetics are also sensitive to temperature changes [19]. Therefore, the absorbance changes of BR at 412 nm have been tested over the whole duration of a typical QENS experiment using a laser pulse energy of 8 mJ/cm^2, and a laser repetition time of 400 ms (see red line in Fig. 1). Since no difference in the kinetics was observed over a period of 6 hours (not shown), the laser-induced QENS data are not affected by effects owing to bleaching of BR nor by a laser-induced (temporary) temperature increase. In order to achieve sufficient statistical accuracy in the time-resolved QENS measurements, however, data from a total of 10 samples (each measured for 6 h) were added up.

(Light-excited) QENS experiments were performed at the time-of-flight spectrometer NEAT (HMI Berlin, Germany) using the optimum laser-excitation conditions defined above. The incident neutron wavelength was 5.1 Å with an elastic energy resolution of 93 μeV. This configuration provides access to diffusive protein motions with relaxation times shorter than ~20 ps. The spectra are averaged over all scattering angles so that $Q = 1.5$ Å^{-1}.

QENS spectra of PM were obtained in three experimental regimes (see below for details): (A) in the dark, (B) with laser-excitation, but without time-selection and (C) selectively during the *M* intermediate after laser excitation. The corresponding data sets measured as a function of energy transfer ω and momentum transfer Q are described by the fit functions $S_A(Q, \omega)$, $S_B(Q, \omega)$, and $S_C(Q, \omega)$, respectively, for localized diffusive and vibrational protein motions (see, e.g., [14]):

$$S_{A,B,C}(\mathbf{Q}, \omega) = e^{-\langle u^2\rangle Q^2}\left\{A_0(\mathbf{Q})\delta(\omega) + \sum_n A_n(\mathbf{Q})L_n(H_n, \omega)\right\} + S_{in}(\mathbf{Q}, \omega). \quad (1)$$

This theoretical scattering function consists of a $\delta(\omega)$-shaped elastic component and a sum of Lorentzians $L_n(H_n, \omega)$ with half widths at half maximum (HWHM) H_n. The fractional intensities $A_0(Q)$ and $A_n(Q)$ are also referred to as elastic and quasielastic incoherent structure factors (EISF and QISF), respectively, while $e^{-\langle u^2\rangle Q^2}$ is the Debye-Waller factor with a vibrational mean square displacement $\langle u^2\rangle$. A satisfactory fit of the PM QENS spectrum in the dark [see $S_A(Q, \omega)$ in Fig. 2] requires two quasielastic components representing localized diffusive protein motions with mean relaxation times $\tau_1 = 12.2$ ps and $\tau_2 = 1$ ps, respectively. The inelastic line shape $S_{in}(Q, \omega)$ is given by a damped harmonic oscillator (DHO) function with an energy of 6.5 meV and a damping of 10 meV similar to that used in [3]. In the following the light-induced QENS spectra will be described by variation of only the EISF and QISFs of the two quasielastic components and of $\langle u^2\rangle$ as visualized in Table I.

A light-induced QENS spectrum of PM was obtained selecting two instants during the presence of the *M* intermediate of BR upon laser illumination (red squares in Fig. 2). The temporal positions of the probe pulses are indicated by red squares in Fig. 1. A comparison to the PM spectrum measured in the dark (black squares in Fig. 2) shows a statistically significant deviation in both, the quasi-

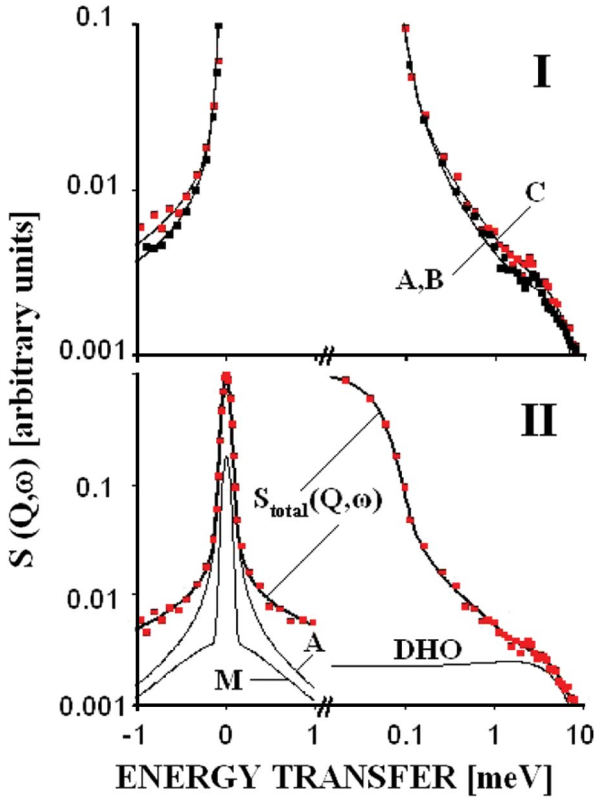

FIG. 2 (color). QENS spectra of wild-type PM hydrated at 98% r.h. D_2O measured with NEAT using an incident neutron wavelength of 5.1 Å and an elastic energy resolution of 93 μeV at room temperature (295 K). The energy transfer axis is composed of a linear (left) and a logarithmic part (right side). Frame I shows a comparison of the PM QENS spectra obtained in the dark (black squares) and selectively during the presence of the M intermediate (red squares), the full lines are fits of the data. Labels A, B, and C correspond to the fit functions $S_A(Q, \omega)$, $S_B(Q, \omega)$, and $S_C(Q, \omega)$, respectively. In Frame II the fit function $S_{\text{total}}(Q, \omega)$ of the light-induced PM QENS spectrum obtained during the M intermediate is composed of two components: (a) a fraction of 80% of the BR molecules remaining in the dark [0.8 $S_A(Q, \omega)$] and (b) an experimentally determined fraction of 20% of the BR molecules in the M intermediate after absorption of a light quantum [0.2 $S_M(Q, \omega)$]. The line shape of the vibrational contribution to the QENS spectra is labeled with "DHO."

and inelastic part, of the QENS spectrum. This is reflected in the model scattering function $S_C(Q, \omega)$ by (i) an increase of the QISF of the fast diffusive protein motions on the expense of the slower component while the EISF remains virtually unaffected, and (ii) an increase of $\langle u^2 \rangle$ (Table I). The reversibility of the light-induced effect can be tested by adding up the QENS spectra of all neutron probe pulses obtained after laser excitation (for temporal positions see diamonds in Fig. 1). The integral contribution of the M intermediate to this spectrum can be estimated to be only ~1.5%. This data set (not shown) was fit by $S_B(Q, \omega)$ which adopted virtually the same parameters as that used for PM in the dark, i.e., $S_B = S_A(Q, \omega)$. Therefore, the changes induced by laser excitation appear to be completely reversible for the excitation conditions applied in the present experiment. Especially, the analysis does not yield any indication for a (global) increase of the sample temperature nor for a sample denaturation. Note, that all data sets were obtained with the same samples measured with and without time selectivity so that the experimental conditions and the applied data correction procedures are exactly the same.

TABLE I. Parameter values of fits shown in Fig. 2.

	QISF$_1$, $\tau_1 = 12.2$ ps	QISF$_2$, $\tau_2 = 1$ ps	$\langle u^2 \rangle$ [Å^2]
$S_A(Q, \omega)$	0.237	0.072	0.08
$S_B(Q, \omega)$	0.237	0.072	0.08
$S_C(Q, \omega)$	0.190	0.119	0.09
$S_M(Q, \omega)$	0	0.309	0.13

Nevertheless, the light-induced QENS spectrum shown in Fig. 2 cannot be directly identified with that of the M intermediate of BR. For a PM sample with an absorbance of 3 at 568 nm, the maximum absorbance difference obtained (see Fig. 1) corresponds to approximately 20% of the BR molecules undergoing the photocycle simultaneously. Most of the residual 80% of the BR molecules remain in the ground state, so that their contribution is identical to $S_A(Q, \omega)$ determined for PM in the dark. The remaining variable contribution $S_M(Q, \omega)$ represents PM with BR molecules in the M intermediate. Within this approach, the sum of the QENS spectra measured selectively is given by

$$S_{\text{total}}(Q, \omega) = 0.8 S_A(Q, \omega) + 0.2 S_M(Q, \omega). \quad (2)$$

A fit using this model function is shown in Frame II of Fig. 2. The parameters displayed in Table I reveal that only the fast ps-component remains in $S_M(Q, \omega)$, which is indicative for a significant acceleration of the diffusive protein motions accompanied by an increase in $\langle u^2 \rangle$. In conclusion, light excitation of wild-type PM at room temperature under nearly physiological conditions has led to a transient alteration of both, diffusive as well as vibrational protein dynamics, in the M intermediate of BR, which can be viewed as a "transient softening" of the protein in the M intermediate of BR.

The observation of a modulation of protein dynamics during a functional process in a protein raises the question about the origin and the physiological significance of this effect for the BR photocycle. Here, the pigment retinal is covalently bound via a protonated Schiff's base. A proton is released from the Schiff's base after a light-induced all-*trans* to 13-*cis* isomerization of retinal. The following proton translocation steps and the corresponding force field alterations result in changes in the protein structure. Most prominent is the large-scale structural transition in the M intermediate, when the accessibility of the Schiff's base is

switched from the extracellular to the cytoplasmic side of PM [15,16]. So far, it has been indirectly concluded from a comparison of separate hydration-dependent experiments that protein flexibility acts as a ''lubricating grease'' for the large-scale structural change within the *M* intermediate (see, e.g., Ref. [9]). Based on such results it has always been inferred that the structural changes during the BR photocycle must be accompanied by a modulation of protein dynamics. Nevertheless, a previous attempt [20] to trap the *M* intermediate in a steady-state experiment at 250 K using the D96N-mutant of BR did not yield indications of altered protein dynamics. However, at such low temperatures diffusive protein motions are widely suppressed and small changes may thus be difficult to detect.

In the present experiment the effect of light excitation on protein dynamics is probed for wild-type BR in real time at room temperature under almost physiological conditions. This approach has led to the unambiguous identification of an increase of fast diffusive protein motions and of a significantly larger vibrational mean square displacement correlated with the structural changes in the *M* intermediate of BR. This means that the excitation energy gained from the light quantum absorbed locally at the chromophore has been converted into a general softening of the BR protein. It is thus attractive to assume that light excitation induces a softer environment as a prerequisite to overcome potential barriers during the large-scale structural changes in the *M* intermediate of wild-type BR. Once the temporary softening decays, the structural transition becomes irreversible ensuring an unidirectional proton transport. Such an interpretation is in line with thermodynamic calculations for the BR photocycle, which find the major loss of the free energy acquired from the light excitation in the *M* intermediate [19]. This implies that the functional importance of protein dynamics goes beyond that of a ''lubricating grease'' which accommodates functionally important conformational changes. Rather, protein mobility may play a very specific role in physiological processes requiring large-scale structural changes as demonstrated here for the case of the BR photocycle. Functionally important modulations of protein dynamics as demonstrated here for the membrane protein BR may thus be of relevance for other proteins exhibiting conformational transitions in the time course of functional operation. The developed approach is applicable to all photoactive proteins and all enzymatic systems activated by photolysis of caged compounds. In any case, the transient alteration of protein dynamics is a direct proof for the functional significance of protein conformational flexibility.

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