Powdered G-Protein–Coupled Receptors

Suchithranga M. D. C. Perera[#][†], **Udeep Chawla**[#][†], and **Michael F. Brown**[*,†,‡]

[†]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721, USA

[‡]Department of Physics, University of Arizona, Tucson, AZ 85721, USA

[#] These authors contributed equally to this work.

Abstract

Preparation and storage of functional membrane proteins such as G-protein-coupled receptors (GPCRs) are crucial to the processes of drug delivery and discovery. Here we describe a method of preparing powdered GPCRs using rhodopsin as the prototype. We purified rhodopsin in CHAPS detergent with low detergent to protein ratio so the bulk of the sample represented protein (ca. 72% w/w). Our new method for generating powders of membrane proteins followed by rehydration paves the way for conducting functional and biophysical experiments. As an illustrative application powdered rhodopsin was prepared with and without the cofactor 11-*cis* retinal to enable partial rehydration of the protein with D_2O in a controlled manner. Quasielastic neutron scattering studies using both spatial motion and energy landscape models form the basis for crucial insights into structural fluctuations and thermodynamics of GPCR activation.

Keywords

Detergent; Drug delivery; GPCR; Hydration; Membrane proteins; Quasielastic neutron scattering; Rhodopsin

Membrane proteins comprise more than one-half of the pharmaceutical drug targets.[1–3] The G-protein-coupled receptors (GPCRs) are particularly important, because they are involved in regulation of key physiological processes involving mediation of cellular responses to hormones and neurotransmitters,[4] including vision, taste, and olfaction. Studies of the structure and function of GPCRs are crucial for development of more effective pharmaceuticals, and for understanding the general mechanisms of cellular signaling.[5] One of the great difficulties in studying membrane proteins such as GPCRs is their location within biological membranes. Investigation of GPCRs typically requires extraction from their native lipid environment, and functional and structural stabilization by embedding them into membrane mimetics like detergent protein-micelles,[6] protein-detergent-lipid bicelles,[7] or protein-lipid nanodiscs.[8] The process of removal from the native physiological environment can be detrimental to membrane protein integrity and stability, even if the process is performed at low temperatures. Here we report a novel method of preserving solubilized fully functional GPCRs through a detergent lyophilization method, which

[*]Correspondence should be addressed to M.F.B. (mfbrown@u.arizona.edu).

potentially increases their shelf life well beyond what one could achieve by mere freezing of the detergent-solubilized protein samples. This new method to stabilize GPCRs forms the basis for a number of applications, and paves the way for applying quasielastic neutron scattering (QENS) methods to powdered rhodopsin.

Lyophilization (also known as freeze-drying) is a technique of dehydrating a sample under a vacuum at cryogenic temperatures; the method converts a solution of a protein sample (at room temperature) to a powder by removing water through the sublimation process. Obtaining the powdered protein sample in this way may enhance the stability of the protein. Moreover, this method of protein preservation has additional advantages over freezing the samples.[9] For instance, during conventional freezing, the protein properties and stability can be affected due to local concentration of the salts of the buffer.[9] When water crystals are formed during the freezing process, the hydrated salt ions are partitioned into the excluded volume at very high concentrations, which in turn can affect the protein properties. By contrast, flash freezing the protein using liquid nitrogen during lyophilization avoids such local concentration of salt ions before sublimation of water to obtain the powdered protein sample. Because the frozen water is removed by sublimation, freeze-drying avoids the potentially detrimental thawing of the sample, which leads to local concentration of salts as in the case of conventional freezing.

In the present application, we investigated whether membrane proteins such as rhodopsin can be prepared as dry powders with either lipids or detergents. The dry protein powders regain their photochemical functionality upon rehydration with bulk excess water. (The high optical density makes it challenging to investigate whether rhodopsin can be light activated in the powdered form, i.e., before rehydration.) For this purpose, we removed the bulk lipids[10] to purify the integral membrane protein rhodopsin (Fig. 1a) in a detergent environment (for details see supplementary information, SI). We chose 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent with an aggregation number[11] of ca. 10 to achieve the minimum protein to detergent molar ratio (27:1) (ca. 72 % w/w protein) in the samples. Bovine retinal disk membranes (RDM) were solubilized in CHAPS detergent, and purified using a zinc-extraction method.[10] The rhodopsin purified in CHAPS had a purity (A_{280}/A_{500}) ratio in the range of 1.7–1.8 and a yield of about 60% (w/w) (percentage purified rhodopsin recovered from RDM (see SI for details). The nominal amount of CHAPS detergent in the solid sample was about 25% (w/w), and was adjusted using ultracentrifugal filters of 30-kDa molar mass cut-off. Thereafter, the protein-CHAPS detergent solution was subjected to lyophilization by flash freezing at 77 K. Surprisingly, UV-visible spectroscopic characterization of the powdered samples (after rehydrating) by dilution in excess water, keeping the detergent to protein ratio constant (ca. 600 μM CHAPS and 22 μM protein), showed the photochemical functionality of rhodopsin remained unaffected by lyophilization (Fig. 1b). The 500-nm peak observed for the rehydrated powdered rhodopsin-CHAPS samples indicated that the rhodopsin was still in the dark state, and that photoillumination yielded a mixture of inactive metarhodopsin-I and active metarhodopsin-II states.[12] This is surprisingly true for both rhodopsin in powdered disk membranes (Fig. S1) and powdered rhodopsin-CHAPS detergent preparations upon rehydration (Fig. 1b). Moreover, far-UV circular dichroism (CD) spectra acquired for the powdered rhodopsin-CHAPS samples and powdered opsin-CHAPS samples when

redissolved in water (ca. 20 μM protein) revealed that the helicity was inideed conserved as expected (Fig. 1c).[13–16]

Further applications potentially include drug design,[17–18] dry powder inhaling,[19] studies of GPCR signaling mechanisms,[20] solid state NMR[21–23] and spin-label EPR[24–25] spectroscopy, and neutron scattering studies.[26] Here, we show the use of the powdered GPCR preparation method to investigate rhodopsin versus the ligand-free opsin apoprotein. Rhodopsin is a class A GPCR responsible for vision under dim light conditions in vertebrates. It is the canonical prototype of the *Rhodopsin* family of GPCRs. The chromophore 11-*cis*-retinal locks the rhodopsin in the inactive dark state,[27] where it acts as an inverse agonist by preventing the interaction with its cognate G-protein (transducin). Activation of the model GPCR rhodopsin is controlled by the chromophore in the ligand-binding pocket of the receptor. Upon photon absorption, the 11-*cis*-retinal isomerizes to all-*trans*, yielding rearrangement of the protein conformation by two protonation switches.[28–29] Photoisomerization of retinal occurs within less than 200 fs,[30] causing rhodopsin to undergo a series of multiscale conformational transitions,[31–32] where dynamics of the protein play a crucial role in its biological signaling function. Solid-state NMR methods,[21, 33] X-ray diffraction,[34] solution X-ray scattering,[35] and site-directed spin labeling (SDSL)[36] have all been extensively applied to study the functional reaction cycle of rhodopsin.

Current understanding of pharmacologically important GPCRs such as the serotonin, β-adrenergic, or angiotensin receptors[5, 37] suggests that protein dynamics hold the key to understanding their functions. Conformational fluctuations of the protein upon extracellular stimulation lead to the activation of GPCRs in a cellular membrane lipid environment. Notably, X-ray crystallographic experiments[38] and recent time-resolved wide-angle X-ray scattering studies[35] conducted on the prototypical visual GPCR rhodopsin have revealed valuable information about the conformational changes that occur during activation. X-ray crystal structures are presently available for rhodopsin in the dark state,[39] as well as several freeze-trapped photointermediates,[38, 40] including the ligand-free opsin apoprotein. However, thus far little information is available regarding how the internal dynamics of the protein change during GPCR activation.[41] Application of the powdered GPCR preparation method together with use of neutron scattering techniques allows for the study of changes in the protein dynamics upon rhodopsin activation. Current findings suggest the intrinsic dynamics of the protein are unlocked by the light-induced isomerization of the 11-*cis* retinal cofactor needed for interaction of the GPCR with its cognate G-protein (transducin).[26]

As an illustration, both elastic and quasielastic neutron scattering methods have been applied to rhodopsin as a canonical GPCR prototype.[26] A combination of the novel powdered rhodopsin sample preparation and its application to quasielastic neutron scattering (QENS)[42] provides insights into the hydrogen-atom dynamics in dark-state rhodopsin versus the ligand-free opsin apoprotein.[26] Backscattering spectrometers such as BASIS[42] open new possibilities and opportunities of extending this technology to complex and biologically relevant systems such as membrane proteins (Fig. 2). The new sample preparation method plus advances in QENS technology were applied to directly probe the effect of the retinal cofactor on the dynamics of rhodopsin in the β-fluctuation time range crucial for its activation. For the QENS applications, protein samples are required with the exchangeable

protons replaced with deuterons, and controlled hydration of the samples with D_2O. Therefore, it is imperative to remove all the bulk water to produce dry rhodopsin samples via lyophilization. As a control, we first lyophilized rhodopsin in native disk membranes (RDM) (see SI), and the resulting lyophilized powder was resuspended with distilled water and characterized. We found that lyophilization does not affect the purity (A_{280}/A_{500} spectral ratio) of the retinal disk membranes (RDM) (Fig. S1).

Notably, for proteins the QENS method is used to study the translational, rotational, and diffusive motions involving hydrogen atoms, due to the polypeptide backbone, methylene, and methyl groups.[43] The incoherent neutron scattering technique is optimal for studying the dynamics of biological molecules, as they mainly contain hydrogen atoms, which have the largest incoherent scattering cross-section for neutrons.[44] Quasielastic neutron scattering methods enable one to probe molecular motions from picoseconds up to nanoseconds within the atomic to molecular length scales.[26] Upon hydrating the protein sample with D_2O, the results of a QENS experiment are expressed as the dynamic structure factor, which is dominated by the contributions from non-exchangeable hydrogen atoms in the protein. The QENS data can be analyzed both in the energy domain by a model-free analysis, and in the time domain by introduction of motional models. For instance, a spatial motion model (SMM) can be introduced by applying mode-coupling theory, as originally used to describe the complex dynamics in glass-forming liquids. A highly non-exponential relaxation of the density correlations and single-particle correlation functions is thus observed. Alternatively, an energy landscape model (ELM) can be introduced as described by Frauenfelder et al.,[45] in which there is no separation into elastic and quasielastic lines. Rather, the entire spectrum is inhomogeneously broadened, due to a random walk of the protein among the various tiers of the energy landscape, in which the neutrons are treated as de Broglie wave packets.[45]

Together with QENS techniques the novel powdered GPCR preparation method opens the door to uncovering subtle changes in the protein dynamics due to the retinal cofactor of rhodopsin.[26] For D_2O-hydrated powders of rhodopsin/CHAPS detergent, the majority of the recorded signal is due to the protein dynamics, and the correction for the presence of the detergent is minimal during the subsequent data reduction and analysis. The QENS experiments were focused on studying how the dynamics of the protein were affected upon rhodopsin photoactivation. Protein dynamics of the dark-state rhodopsin were compared to those of ligand-free opsin,[26] which is structurally similar to active metarhodopin-II.[38] Previous neutron scattering studies conducted to unravel the local dynamics of bacteriorhodopsin and visual rhodopsin in the dark and light-activated states have revealed few significant differences.[46–47] Our optimization of the sample preparation (see above) for the QENS experiment and methods refinements[48] made it possible to capture the elusive protein dynamics during rhodopsin activation, which was not possible previously.

Neutron scattering spectra are conventionally interpreted by a spatial motion model (SMM) in terms of separate elastic and quasi-elastic processes.[26] The QENS spectrum is separated into an elastic component (elastic line at $E = 0$), and a quasielastic component (broadened wings of the spectrum) treated as a Lorentzian centered around $E = 0$ (elastic line). In the SMM analysis, the broadening of the QENS spectrum is ascribed to spatial motion of the probed atoms. Application of the SMM in the time domain yields β-relaxation times (τ_β)

that correspond to the local structural fluctuations of the protein.[26] Further analysis of the QENS spectra probes the relaxational dynamics of hydrogen atoms in various length scales of the protein molecule, including vibrations, local β-fluctuations, and collective α-processes. Applying the spatial motion model to the QENS data obtained for the powdered dark-state rhodopsin and ligand-free opsin samples suggests crucial insights into the role of the retinal cofactor in regulating protein mobility.[26] The SMM implies the characteristic τ_β relaxation time calculated for the ligand-free opsin is substantially longer versus the dark-state rhodopsin. A possible explanation is that the opsin apoprotein is softer than dark-state rhodopsin.[26] Yet Arrhenius plots reveal the activation energies associated with the local fluctuations in the dark-state rhodopsin and the ligand-free opsin are similar.[26] Differences between the relaxation rates can be explained by the Arrhenius coefficient (τ_0) (pre-exponential factor), which includes steric effects on the local hydrogen-atom dynamics. The slower relaxation rate in opsin also suggests the possibility of greater steric crowding, that retards the hydrogen-atom dynamics within the β-relaxation time regime versus dark-state rhodopsin. Slowing down the hydrogen atom dynamics would be consistent with under-hydration of the opsin conformation versus dark-state rhodopsin.[26] This increased steric hindrance for local fluctuations in opsin is reflected by an increase in the pre-exponential factor (τ_0), in the Arrhenius equation, yielding slower local dynamics in opsin versus dark-state rhodopsin.

Increased steric crowding by collapsing of the protein structure for opsin versus dark-state rhodopsin could result from removal of the retinylidene cofactor. Formation of opsin by dissociation of the all-*trans* retinal from metarhodopsin-II entails two forms: the more open low-pH form (Ops*) with basal activity and the closed inactive opsin (Ops) state. The Ops* form is similar to the active metarhodopsin-II structure, whereas Ops is believed to be structurally similar to dark-state rhodopsin.[27, 49] A more collapsed protein structure for opsin compared to dark-state rhodopsin would be consistent with elevated mechanical rigidity of ligand-free apoprotein as reported previously.[50] Recently, the conventional separation into distinct elastic and quasi-elastic bands has been called into question by Frauenfelder *et al.*,[45] who instead propose an energy landscape model (ELM). The QENS spectrum is thus composed of multiple individual spectral lines corresponding to the conformational substates of the protein. The entire QENS spectrum is considered to be inhomogeneously broadened, e.g., by a random walk of the protein molecule among the various tiers in the energy landscape (EL) due to the conformational substates. This wave-mechanics based model considers the incident neutrons as de Broglie wave packets that exchange energy with the protein during their encounter. Because the energy exchange depends on the conformational substates, the ELM approach scans the free energy landscape of rhodopsin activation, as well as the structural fluctuations in the protein. An increase of temperature broadens the quasielastic spectrum (Fig. 2), corresponding to an increase in the number of conformational substates occupied by the protein. Interpretation of the QENS spectra using the ELM[45] also supports a more collapsed protein structure in opsin (Ops) versus the dark-state rhodopsin. The overall narrowing of the QENS spectra[26] suggests s reduced ensemble of conformational substates that exchange energy with the incoming neutron wave packets in ligand-free opsin versus dark-state rhodopsin.

In summary, a novel powdered GPCR sample preparation procedure in detergent has been developed for the first time. A high rhodopsin:detergent ratio was the key to the applications reported herein. The powdered GPCR-detergent complex preparation removes water from the samples completely, and enables the subsequent controlled hydration with D_2O by isopiestic transfer through the vapor phase. The UV-visible characterization after rehydrating the powdered samples shows that even one-year after sample preparation, the proteins are still photochemically functional. This new method of preparing functional D_2O-rehydrated powders of rhodopsin may be applied to other GPCRs in the future, thus demonstrating the proof of concept. An important question remaining for future research is whether the powdered GPCR method is applicable to other members of the *Rhodopsin* superfamily of G-protein–coupled receptors.

Materials and Methods

Powdered membrane protein samples containing 72% (w/w) of bovine rhodopsin and 28% (w/w) of CHAPS (3-[(3 Cholamidopropyl) dimethylammonio]-1 propanesulfonate)) detergent were prepared. About 600 mg of the powdered rhodopsin sample was divided into two separate containers, one for preparing a dark-state rhodopsin sample, and other for the ligand-free opsin apoprotein. The opsin was prepared by photobleaching the dark-state sample with a locally constructed 515-nm LED light source. Far-UV circular dichroism (CD) spectra were collected for the dark-state rhodopsin and opsin samples, and confirmed that the helicity is conserved after the lyophilization and rehydration (Fig. 1c). The samples were hydrated with D_2O inside a glove box, with a hydration level of $h \approx 0.27$ (g D_2O/g of protein) (ca. 600 water molecules per rhodopsin molecule) and were enclosed in aluminum foil to prevent exposure to light. Finally, the samples were inserted in a rectangular aluminum sample holder for the near-backscattering spectrometer (BASIS) used for the neutron scattering experiments.[26]

Preparation of retinal disk membranes

All procedures were carried out under dim red light (11-W Bright Lab™ Universal Red Safelight bulb, CPM Delta1, Inc., Dallas, TX), at 4 °C. Retinal disk membranes (RDM) containing rhodopsin were isolated from bovine retinas as described.[41] The RDM pellet was resuspended in 15 mM sodium phosphate buffer, pH 6.9, and characterized using UV-visible spectroscopy[51] to determine the level of purity (A_{280}/A_{500} absorption ratio was typically 2.4). The rhodopsin concentration was estimated from the absorbance of the sample at 500 nm.[29] The sample was photobleached with green LED light of 515 nm, which activates rhodopsin to form metarhodopsin-II.[41, 51] Including NH_2OH ensures complete photobleaching of the dark-state rhodopsin, and is confirmed by the nearly zero absorbance at ca. 500 nm (Fig. S1) (see SI for further details on preparation and characterization of powdered RDM samples).

Preparation of CHAPS-solubilized protein samples

Retinal disk membranes (RDM) with a final rhodopsin concentration of 400 μM were dissolved in 15 mM sodium phosphate buffer, pH 6.9, containing 100 mM CHAPS, and 100 mM zinc acetate, and incubated for 30 min at 4 °C.[10] The hydrophobic part of the detergent

interacts with the protein hydrophobic surface, while the hydrophilic part interacts with the polar solvent thus solubilizing the membrane protein. The solubilized rhodopsin was centrifuged at 24,000 × g (Sorvall SS-34 rotor) for 30 min. Next, the solution was diluted with two volumes of 15 mM sodium phosphate buffer, pH 6.9, and centrifuged at 24,000 × g (Sorvall SS-34 rotor) for 30 min to remove non-solubilized membranes. The supernatant was characterized using UV-visible spectroscopy to determine the quantity and purity. The CHAPS-solubilized rhodopsin was diluted with 15 mM sodium phosphate buffer, pH 6.9, to adjust the detergent to rhodopsin molar ratio to be 27:1 (72% w/w protein). To prepare the powdered opsin sample, 1% (w/v) hydroxylamine was added to 30 mg/mL rhodopsin in 50 mM CHAPS containing 15 mM sodium phosphate buffer solution, and completely photobleached using a 515-nm LED lamp, before starting the lyophilization step. The presence of hydroxylamine ensures that hydrolysis of the Schiff base linkage between retinal and Lys[296] occurs upon photoillumination.[51] The resulting hydrolysis of the retinal from the rhodopsin yields the apoprotein, opsin.[12] About 300 mg (rhodopsin alone) was used for preparation of the opsin sample. A total of ca. 1000 mg of rhodopsin in RDM was solubilized to obtain the powdered dark-state and opsin samples used for the neutron scattering application.[26]

The protein samples were lyophilized as follows (for details see SI). First, the CHAPS detergent-solubilized protein samples were flash frozen at 77 K using liquid nitrogen. Next the flash-frozen samples were subjected to a vacuum of 100 mTorr for 12 hrs in a homemade Schlenk line setup. This step was carried out in a dark room to avoid light exposure of the dark-state rhodopsin samples. Finally, after ca. 12 hrs the powdered protein samples were transferred into Falcon 15-mL centrifuge tubes for storage at −80 °C. A portion of the powdered sample was rehydrated with excess H_2O for UV-visible characterization. For the applications to quasielastic neutron scattering, it is necessary to replace all the exchangeable protons with deuterons in the protein sample. Powdered samples prepared as described above were rehydrated with D_2O and lyophilized again. This step (lyophilization with D_2O) was repeated 3x to ensure the exchange of protons with deuterons. The final dry powdered rhodopsin and opsin samples were hydrated to 27% (w/w) using 99.9% D_2O (Aldrich Chemistry, St Louis, MO) in a glove bag filled with argon gas, by placing a warm 99.9% D_2O bath inside. Finally, the hydrated sample was transferred to the aluminum cell, screwed tight, and inserted into the near backscattering spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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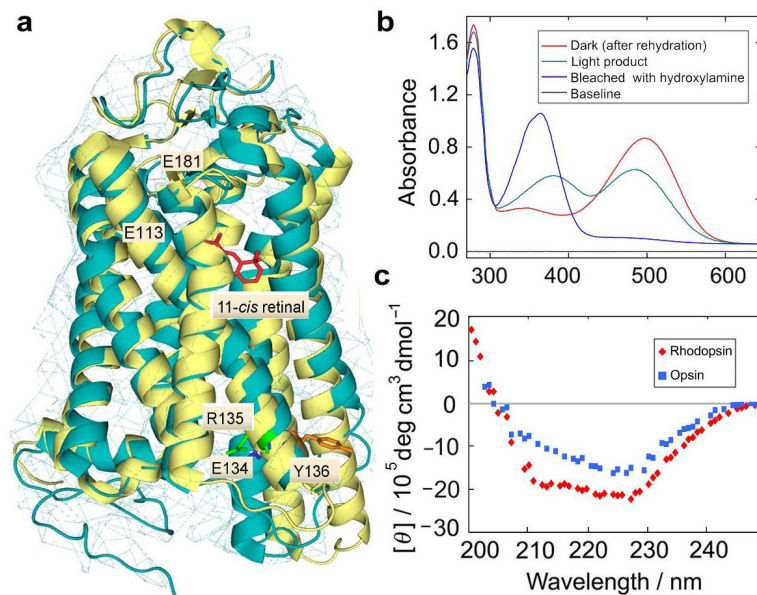
Figure 1.
Powdered rhodopsin-CHAPS detergent complex retains functionality upon rehydration. (a) Dark-state rhodopsin crystal structure (1U19)[39] overlaid on ligand-free opsin crystal structure (PDB code 3CAP).[52] (b) UV-visible spectra for rhodopsin-CHAPS complex after lyophilization and re-hydration with excess H_2O (keeping the detergent to protein molar ratio constant). Results are shown for the dark state, upon light activation, and for fully bleached opsin. (c) Far-UV circular dichroism spectra for powdered rhodopsin and powdered opsin after rehydration in excess H_2O. Upon rehydration samples contained ca. 22 μM rhodopsin in ca. 0.6 mM CHAPS in 15 mM sodium phosphate buffer (pH 6.9), and spectra were collected at 15 °C.
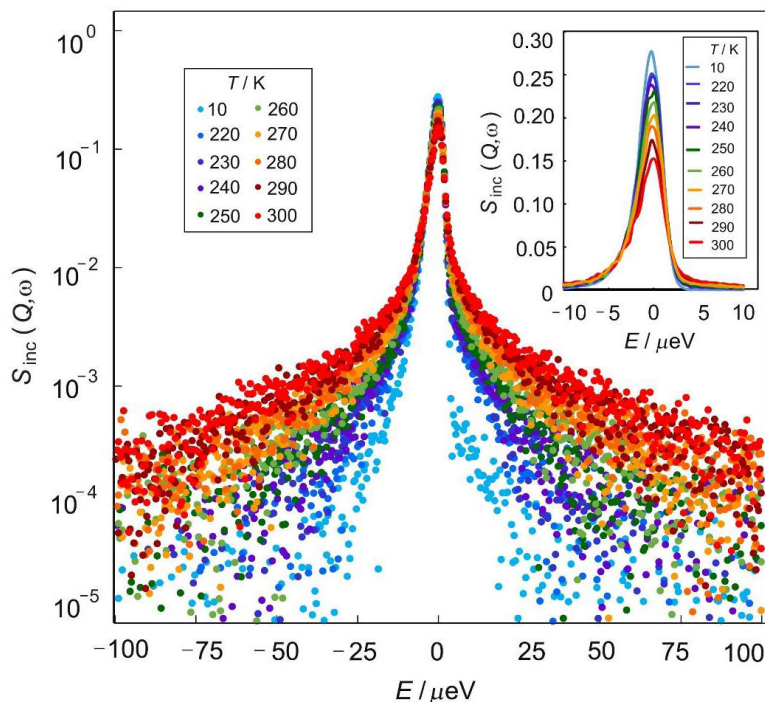
Figure 2.
Increased temperature yields reduction in elastic scattering with greater quasielastic broadening in QENS spectra. Normalized dynamic incoherent scattering function, $S_{inc}(Q,\omega)$ plotted as a function of energy transfer (E) for dark-state rhodopsin at temperatures varying from T = 220 K to 300 K for Q = 1.1 Å^{-1}. The QENS spectra were normalized to unity over the measured range of transfer energy from −120 to +120 μeV. The instrument resolution function was collected at 10 K and is plotted in light blue. Inset: Expansion of elastic component at various temperatures. Note that dark-state rhodopsin has a reduction in the elastic scattering and increased quasielastic broadening [of the spectral wings] as temperature increases.