THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

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Dynamic Behavior of Oligomeric Inorganic Pyrophosphatase Explored by Quasielastic Neutron Scattering

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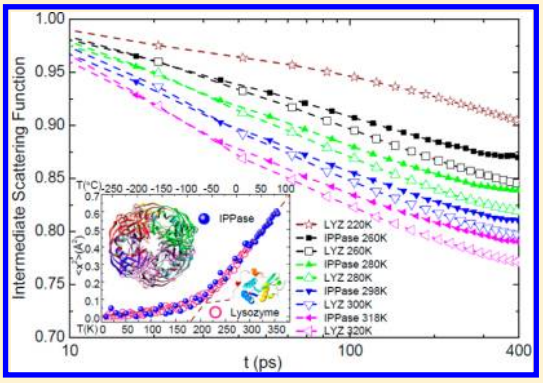
Supporting Information

ABSTRACT: The purpose of this investigation is to determine whether a large oligomeric protein, inorganic pyrophosphatase (IPPase) from *Thermococcus thioreducens* with quaternary structural complexity, would have distinguishable dynamic characteristics compared to those of the small simple monomeric model protein, lysozyme. In this study, the β-relaxational dynamics of the two proteins, IPPase and lysozyme, are compared in the 10 ps to 0.5 ns time interval using quasi-elastic neutron scattering (QENS). Both of the protein dynamics show a characteristic logarithmic-like decay in the intermediate scattering function (ISF) of the hydrogen atoms. Distinguishable dynamical behavior found between two proteins reveals local flexibility and conformational substates unique to oligomeric structures. Moreover, the temperature dependence of the mean square displacement (MSD) of the hydrogen atoms in protein molecules, which is a traditional way to determine the "softness" of the protein molecule, is measured and shows no difference for the two proteins.



INTRODUCTION

Structural biology involves deciphering the spatial atomic arrangements of macromolecules and determining their structural implications on biological function. The three-dimensional structures of proteins deciphered by X-ray or neutron crystallography offers a single snapshot view, at high atomic resolution, of average molecular configurations. However, to truly understand a protein's action and the molecule's complete description, one must also consider the dynamic contribution affected by the protein's different conformational states and the energy barriers between them.

Quasielastic neutron scattering (QENS) has been a prevailing spectroscopic method to measure the biophysical properties from which protein dynamics can be inferred.[1,2] The technique resolves the change in kinetic energy derived from limiting inelastic collisions between neutrons and the nuclei in the sample. The results are expressed as the dynamic structure factor, predominantly of the hydrogen atoms in the protein or the water molecules in the protein hydration layer.[3,4] Since protein structures are exceptionally sensitive to the interactions of their hydrogen bonds with hydration water, the diffusive dynamics of hydrated proteins measured by QENS reveals important information on the protein's global and local conformational flexibility, and, in turn, its functionality.[5]

In this investigation, the hyperthermophilic homohexameric enzyme, inorganic pyrophosphatase (IPPase, EC 3.6.1.1) from *Thermococcus thioreducens*, is studied to determine whether a large oligomeric protein would share dynamic properties measured by QENS, similar to those measured for previously studied proteins with less quaternary structural complexity. Enzymes derived from thermostable microorganisms are of extreme interest for biophysical studies because of their inherent chemical and thermal stability and high temperature activity. IPPase catalyzes the hydrolysis of inorganic pyrophosphate (PP_i) to orthophosphate (P_i). This is a highly exergonic reaction that can be coupled to energetically unfavorable biochemical transformations such as lipid metabolism and nucleic acid polymerization. The quaternary structure of IPPase is a homohexamer with an oligomeric molecular weight of approximately 120 kDa (each subunit is about 20 kDa molecular weight).[6,7] It is the largest enzyme complex studied to date by QENS for structural dynamic characterization. In comparison, lysozyme is the smallest enzyme investigated by QENS and it is a simple monomer with a molecular weight of 14 kDa. The temperature dependent activity of IPPase is shown in Figure S1 (Supporting Information). The optimal temperature for IPPase enzymatic activity was determined to be 358 K (85 °C) compared to that of hen egg lysozyme (HEWL) at 323 K (50 °C), as reported by Hikima et al.[8] In this study, to decipher protein dynamic differences as a function of

Received: April 2, 2012
Revised: June 8, 2012
Published: July 17, 2012

dx.doi.org/10.1021/jp303127w | J. Phys. Chem. B 2012, 116, 9917−9921

quaternary structural complexity, IPPase was compared directly to lysozyme by QENS measurements. Moreover, the relaxational dynamics of the enzymes are measured and compared to its optimal temperature of bioactivity.

MATERIALS AND METHODS

The sample preparation for IPPase is described in detail in the Supporting Information. The final hydration level was determined to be 0.37 g of D_2 per 1 g of IPPase (h = 0.37) corresponding to almost an approximate monolayer of heavy water covering the protein surface.[9] Purified and crystallized hen egg white lysozyme (HEWL, EC 3.2.1.17) used in this experiment was purchased from Fluka (catalogue number: L7651) and used without additional purification. Prior to QENS experiments, the protein was lyophilized overnight to remove residual water followed by D_2O hydration as described in Chu et al.[10] The final hydration level was determined to be the same as that prepared for IPPase.

The near-backscattering spectrometer BASIS[11,12] at the Spallation Neutron Source (SNS) at Oak Ridge National Laboratory (ORNL) was the primary instrument used for the dynamics studies of both IPPase and lysozyme. This spectrometer has an energy resolution of 3.4 μeV (full-width at half-maximum, for the Q-averaged resolution value) and a dynamic range of ±100 μeV. QENS data was collected over a temperature range of 260 K (−13 °C) to 353 K (80 °C). Measurements were made on D_2O-hydrated proteins of both IPPase and lysozyme using the same instrument and the same energy window in order to facilitate the comparison. Because of the exceptionally large neutron incoherent scattering cross section of hydrogen, the scattering signals are dominated by the contribution of the incoherent scattering from the hydrogen atoms of the protein molecules.

RESULTS AND DISCUSSION

1. Quasi-Elastic Neutron Scattering (QENS) Results. Figure 1 shows the measured neutron scattering spectra obtained by QENS at a series of temperatures for hydrated IPPase (A) and lysozyme (B), respectively. These spectra give the self-dynamic structure factor $S_H(Q,\omega)$, which represents the Fourier transformation of the product of the intermediate scattering function (ISF) $F_H(Q,t)$ of the hydrogen atom of the protein molecule and the resolution function in the time space. The broadening of the central peaks from the resolution function indicates the quasi-elastic scattering of neutrons from the hydrogen atoms in the sample. As temperature increases, the central peaks become broader, which implies the faster dynamics of hydrogen atoms in the protein molecules.

In Figure 2, $S_H(Q,\omega)$ is Fourier-transformed into the ISF $F_H(Q,t)$, as described in the Supporting Information, plotted as a function of time. The ISF is referred to as the density−density time correlation function of a single hydrogen particle in the protein molecule measured by neutron scattering experiments. It is useful in describing the relaxational processes in the dynamics of the protein molecule and is the primary quantity of theoretical interest related to the experiment. In Figure 2A, the ISF of IPPase for each temperature ranging from 260 K (−13 °C) to 353 K (80 °C) is shown in the time range of 10 ps to 0.4 ns. An apparent logarithmic-like relaxation process is observed in this experimentally measured time range, which has previously been observed computationally[13] and experimentally[10] for lysozyme molecules. The small-amplitude picosecond

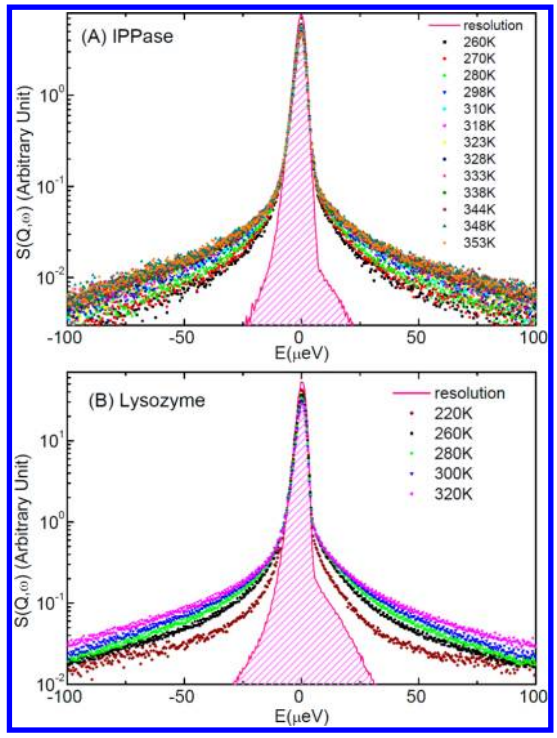


Figure 1. Normalized QENS spectra of (A) hydrated IPPase and (B) hydrated lysozyme after background subtraction at all temperatures, covering the full Q range. The shadowed area represents the instrumental resolution function.

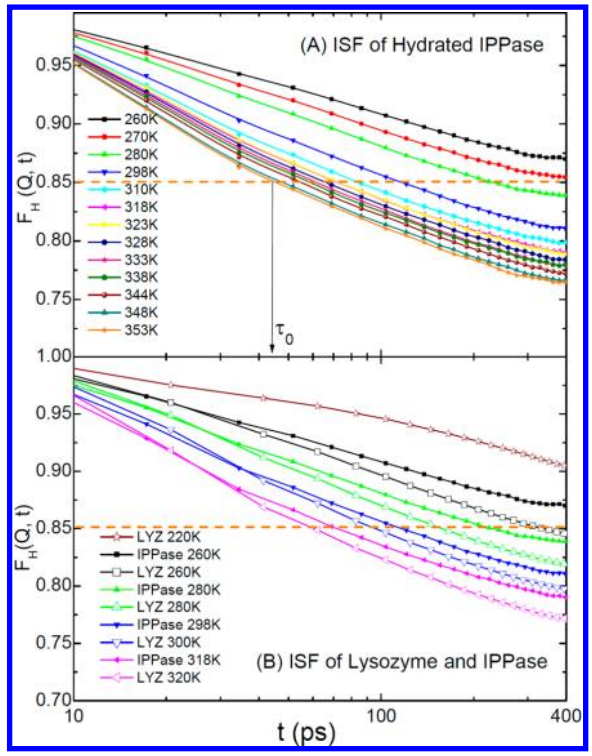


Figure 2. Intermediate scattering function (ISF) of the hydrogen atoms in hydrated IPPase and lysozyme. (A) ISF of hydrated IPPases are plotted at temperature ranges from 260 K (−13 °C) to 353 K (80 °C). (B) ISFs of hydrated lysozyme and IPPase are compared directly at selective temperatures. The open symbols represent data belonging to lysozyme (LYZ), while the solid symbols are those of IPPase.

dx.doi.org/10.1021/jp303127w | J. Phys. Chem. B 2012, 116, 9917−9921

to nanosecond fluctuations (β-relaxation) observed here at physiological temperatures represent an ensemble of structurally similar states separated by energy barriers of less than 1kT.[5]

Similarly, the ISFs of lysozyme at five temperatures are plotted within the same time scale and compared directly against those of the IPPase near the same temperature (Figure 2B). The relaxation time for IPPase is slower than that for lysozyme, where the dynamical behaviors of the two proteins are not identical as detected by elastic scattering (Figure 6). As indicated by the dashed lines at ISF = 0.85, the values of τ_0 are in the ~100 ps range and correspond to the relaxation dynamics of local flexibility contributed by the amino acid side-chain rotamers of the protein. Such dynamic processes are consistently slower in the IPPase compared to those from the lysozyme within our measurement temperature range. However, at shorter times where the values of τ_0 are in the ~10 ps range, both IPPase and lysozyme display similar relaxation times at each temperature. This time range corresponds to somewhat faster dynamics supposedly due to the methyl group rotations.[14] Therefore, the average relaxation dynamics of the side-chain groups, excluding the smallest structural units such as methyl groups, is slower in the IPPase.

In Figure 3, the τ_0 values at ISF = 0.85 are plotted in log scale as a function of inverse temperature, which is the so-called

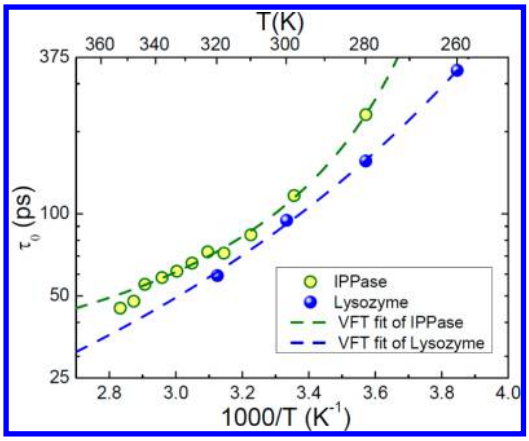


Figure 3. Arrhenius plot of the relaxation time τ_0 at ISF = 0.85 for both IPPase (circles) and lysozyme (spheres). The dashed lines are the VFT (Vogel–Fulcher–Tammann) fits for both proteins.

Arrhenius plot. In the experimental temperature range between 260 K (−13 °C) and 344 K (71 °C), the dynamic behavior of the hydrogen atoms in both IPPase and lysozyme exhibits super-Arrhenius temperature dependence and can be fitted using the Vogel–Fulcher–Tammann (VFT) law, $\tau_0 = \tau_1 \exp[DT_0/(T - T_0)]$, where D is a dimensionless parameter providing the measure of fragility and T_0 is the ideal glass transition temperature. It is known from the literature[15−17] that this particular relaxation of proteins is super-Arrhenius. This is despite the fact that this relaxation is clearly not α-relaxation but should be assigned to β-relaxation.[10,13] This β-relaxation is super-Arrhenius because it is well-known[18−20] to be coupled to the relaxation of its hydration water which is clearly super-Arrhenius.[21−26]

Specifically, as the temperature is raised above 344 K (71 °C) for IPPase, the temperature dependence of τ_0 does not follow the VFT law anymore. It apparently switches over to another dynamic behavior. In the case of lysozyme, there is no measurement above 320 K (47 °C) to avoid thermal denaturation.[27−29] Due to the limited data points measured above 344 K for IPPase, it is hard to determine whether the dynamics shows an Arrhenius behavior above this temperature. However, in a previous study on lysozyme hydration water at high temperatures,[30] a similar dynamic crossover temperature was observed at $T_D = 345 \pm 5$ K for protein hydration water, which has also been proven by MD simulation results.[30]

However, IPPase is much larger molecule and more complex. The oligomerization of IPPase protein proceeds with a major expense of reducing translational and rotational entropy for their monomers in order to form the stabilizing interactions for its final quaternary structure. These include contributions of polar/apolar, hydrophobic, ion pairs, and hydrogen bond contacts between monomers and non-solvent-exposed cavities. Local flexibility that encompasses methyl rotation and loop motion is also distinguished here from midtime dynamics, revealing a more involved energy landscape compared to a simple monomeric model protein such as lysozyme. The average relaxation dynamics of hydrogen atoms within the side chain groups of IPPase molecules is observed to be slower (less mobile) than that of lysozyme at the same temperature (Figures 2 and 3). Optimal catalytic activity may therefore be associated with increased movement of protein-surrounding water molecules that contribute to domain movements necessary for optimal enzymatic activities.

2. Mean Square Displacement Calculated from Elastic Neutron Scattering. On the other hand, protein flexibility, often referred to as the protein "softness",[31] can be determined from the mean square displacement (MSD) $\langle x^2 \rangle$ of a given atom in the protein molecule.[25,32] Traditionally, the slope of the MSD plot as a function of temperature is used as a

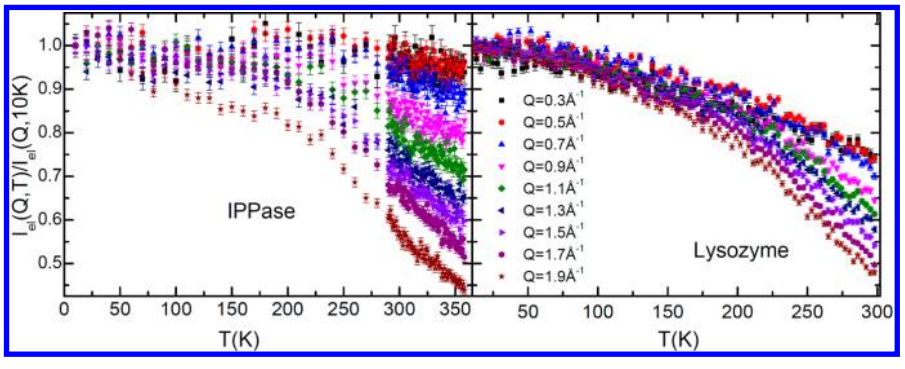


Figure 4. Elastic scattering intensity, $I_{el}(Q, T, \omega = 0)$, normalized to its low temperature limit (10 K) at nine Q values, of IPPase (left) and lysozyme (right), respectively.

dx.doi.org/10.1021/jp303127w | J. Phys. Chem. B 2012, 116, 9917−9921

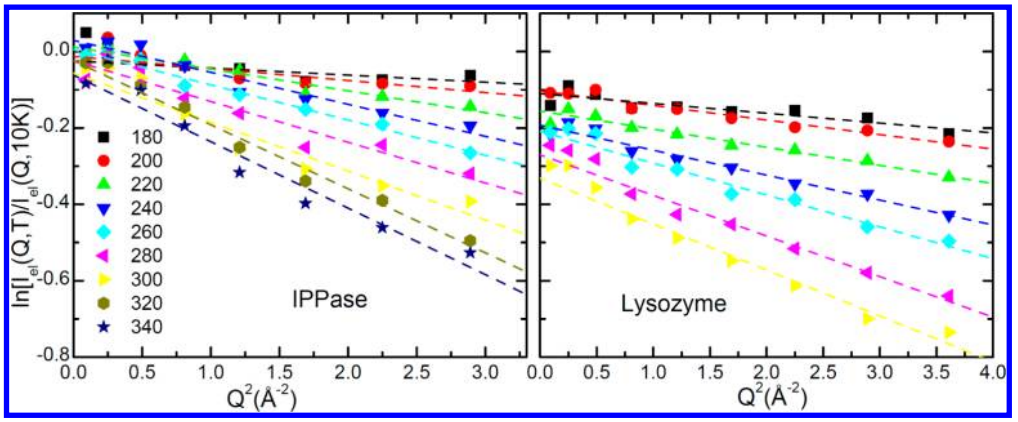


Figure 5. The linear fits according to the Debye–Waller factor, $S_H(Q, \omega = 0) = \exp[-Q^2\langle x^2\rangle]$. Here $\ln[I_{el}(Q, T, \omega = 0)/I_{el}(Q, T = 10\ \text{K}, \omega = 0]$ is plotted as a function of Q^2, and linear fits are using the broad Q range up to $Q^2 \sim 3.5\ \text{Å}^{-2}$.

measurement of the protein's flexibility[31]—the steeper the curve, the softer the biological material is at a given temperature. MSD studies using QENS have been carried out with a handful of proteins including myoglobin,[14,33] maltose binding protein (MBP),[34] RNaseA,[35] hemoglobin,[36] human butyrylcholinesterase (HuBChE),[37] and lysozyme.[25,32,38−40] Almost all of the proteins studied have shown a similar dynamic transition[33,41] in their MSDs at a characteristic temperature of about 220 K at ambient pressure.

The method of extracting the MSDs from the elastic neutron scattering intensity is described in detail in the Supporting Information. To elucidate the validity of our MSD calculation, we plot in Figures 4 and 5 the elastic neutron scattering (ENS) intensities and the linear fitting according to the Debye–Waller factor. Although the ENS intensities are very different for the two proteins, the extracted MSDs (Figure 6) are virtually

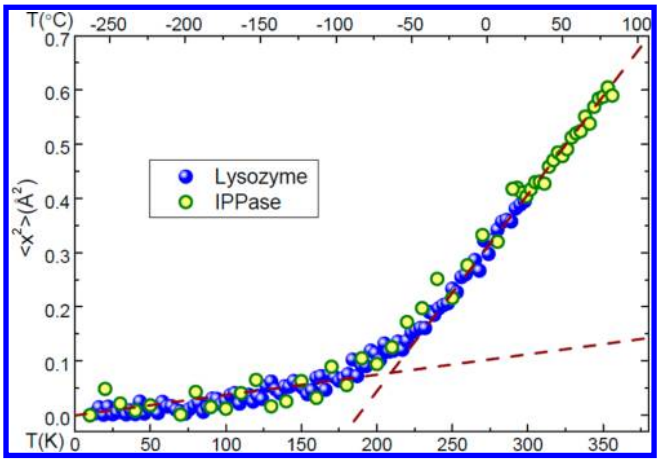


Figure 6. The MSD, $\langle x^2\rangle$, of D_2O hydrated of IPPase is compared to that of lysozyme. A transition temperature of ~220 K (−53 °C) is shown to be in common for both enzymes. The dashed lines indicate the slopes of the MSD vs T curve, which is a qualitative indicator of the softness of the proteins.

identical in the measurement temperature range. From the Gaussian approximation that has been used in deriving the Debye–Waller factor to extract the MSDs, the validity of the Debye–Waller factor is restricted to small Qs. At higher Qs, non-Gaussian effects due to static and dynamical heterogeneity may play an important role.[42,43] A simple simulation-tested model for non-Gaussian elastic incoherent structure factor (EISF) has been proposed,[43] and more recently, a correction for high Q is suggested to be used.[44] However, as one can see in Figure 5, the Gaussian approximation appears to be satisfactory through the entire Q range in our fitting. Although this approximation is typically used for Q values smaller than 1 Å^{-1}, it has been suggested to remain applicable to much higher Q values.[45] Thus, in Figure 5, a Q range up to $Q^2 \sim 3.5\ \text{Å}^{-2}$ is used in the fitting to extract the MSDs. Additionally, the contribution from multiple scattering is usually significant at lower Qs compared to that at higher Qs.[38] Multiple scattering corrections are not a trivial task and complicate the analysis. To eliminate contributions due to multiple scattering in the analysis, we only considered intensities for $Q > 0.5\ \text{Å}^{-1}$.

In Figure 6, the MSDs of both IPPase and lysozyme are measured by BASIS and compared as complementary data of this study. The master curve of the MSD $\langle x^2\rangle$ as a function of temperature of IPPase in the temperature range of 10−300 K appears to be very similar to that of lysozyme. A transition temperature can be clearly seen near 220 K (−53 °C) where the slopes of the MSD plots become steeper, indicative of the increase of protein flexibility.[25,31] It has also been proposed that the rapid increase in the MSD at this temperature may be due to dielectric fluctuations in the hydration shell (as in the case of myoglobin).[14] Even though the optimal enzymatic activities for IPPase and lysozyme differ considerably, the curves corresponding to their MSD as a function of temperature are virtually identical in the measurement temperature range. Since hydration water shows a similar dynamic crossover at around the same temperature,[23,25,46] it is believed that the transition state is prompted by the protein's firm coupling with the hydration water independent of the protein's quaternary structure.

■ CONCLUSION

In summary, the investigation reported here shows a large thermophilic oligomeric protein, IPPase, to have distinguishable dynamic properties measured by QENS from those of a simple monomeric mesophilic protein, lysozyme. The traditional method of taking the mean squared atomic displacement (MSD) as a measurement of protein softness shows no difference between IPPase and lysozyme. Both of them have a universal low temperature dynamic transition coincidental with the crossover temperature of water hydration at 220 K. However, midtime β-relaxation dynamics in the 10 ps to 0.5 ns time interval show distinguishable dynamical behavior between

dx.doi.org/10.1021/jp303127w | J. Phys. Chem. B 2012, 116, 9917−9921

IPPase and lysozyme that may reveal local flexibility of oligomeric structures that are not seen otherwise. The relatively slower relaxation dynamics of the IPPase's side-chain groups can be linked to the higher temperature optimums for catalytic activity when compared to lysozyme. The present study provides important parameters for future extensive studies on dynamic adaptation to oligomeric stability and bioactivity.

■ ASSOCIATED CONTENT

Supporting Information

Sample preparation for IPPase, the activity−temperature profile of IPPase, and neutron scattering data analysis procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research at Oak Ridge National Laboratory's Center for Structural Molecular Biology was supported by the Office of Biological and Environmental Research, using facilities supported by the U.S. Department of Energy, managed by UT-Battelle, LLC, under contract no. DE-AC05-00OR22725. Research at Oak Ridge National Laboratory's Spallation Neutron Source was sponsored by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy. L.C. would like to deeply thank R. C. Hughes for the IPPase recombinant clone and H. M. O'Neill and Q. Zhang for their help with sample preparation.

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