Neutron diffraction studies on rubredoxin from *Pyrococcus furiosus*

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Single-crystal neutron diffraction data up to a resolution of 1.5 Å have been collected at room temperature on two forms of rubredoxin using the BIX-3 diffractometer at the JRR-3 reactor of the Japan Atomic Energy Research Institute (JAERI). Rubredoxin is a small iron-sulfur redox protein with 53 amino acid residues, and the source of this particular protein is the hyperthermophile *Pyrococcus furiosus*, a microorganism that normally lives at temperatures near that of boiling water. Data were collected on crystals of the wild-type protein and on a mutant in which three of the residues have been replaced. In this paper we will be describing several sets of results arising from these high-resolution neutron structure determinations: (a) the H/D exchange pattern of the N-H bonds of the main backbone, which give information about which regions of the molecule are more exposed to solvent; (b) the orientations of some of the O-D bonds in the protein, information which is often not obtainable from X-ray results; (c) the structure and appearance of water molecules in the protein crystals; and (d) some structural features which may help rationalize the remarkable thermal stability of the wild-type protein from this intriguing microorganism.

Keywords: neutron diffraction; rubredoxin; pyrococcus furiosus; hydrogen bonds; hydrogen-deuterium exchange.

1. Introduction

In an attempt to understand the exceptional thermostability of rubredoxin from the hyperthermophile *Pyrococcus furiosus* (Jenney *et al.*, 2001), we embarked upon the neutron diffraction analysis of its three-dimensional structure, in the hope that a detailed knowledge of the hydrogen positions in this small protein would reveal additional geometrical details that were not apparent from an earlier high-resolution X-ray structure determination (Bau *et al.*, 1998; Day *et al.*, 1992). Additionally, we wanted to test the capabilities of the BIX-3 diffractometer (Tanaka *et al.*, 1999, 2002; Niimura *et al.*, 1994) at the Japan Atomic Energy Research Institute (JAERI), which at the time was just starting to come into operation. We describe here some details of the resulting 1.5Å-resolution neutron diffraction study, as well as a subsequent structure determination of a "triple mutant" variant of the wild-type protein. More importantly, we wish to illustrate in this manuscript the usefulness of neutron diffraction to solve certain problems of biological interest, and we will highlight here a few examples of the types of information that can be obtained with this powerful technique.

2. Experimental section

Rubredoxin (RdPf) from *Pyrococcus furiosus*, a hyperthermophilic microorganism that grows in deep undersea vents (Verhagen *et al.*, 2001; Zierenberg & Adams, 2000; Stetter, 1998), was expressed and purified by the group of M.W.W. Adams of the University of Georgia, using a procedure previously described in the literature (Jenney *et al.*, 2001; Day *et al.*, 1992). Crystals large enough for single-crystal neutron diffraction analysis were grown from D_2O by the sitting-drop vapor-diffusion procedure, using 3.8 M NaK phosphate (equimolar NaH_2PO_4/K_2HPO_4) as the precipitant (Bau *et al.*, 1998; Kurihara *et al.*, 2001, 2003). Neutron data collection on the wild-type protein using the BIX-3 diffractometer at JAERI was carried out on a crystal about 5 mm^3 in volume and took approximately 30 days (Kurihara *et al.*, 2001, 2003). The other sample of rubredoxin used in this study (hereafter called the "triple mutant", mut-RdPf) involved replacing (Jenney *et al.*, 2001) three key amino-acid residues in the core of the protein (specifically, Trp3, Ile23 and Leu32) with the corresponding residues (Tyr3, Val23 and Ile32) from a mesophilic rubredoxin (one from *Clostridium pasteurianum*, a bacterium that grows at normal temperatures). Crystals were grown and neutron data were collected (Chatake *et al.*, 2003) in a completely analogous manner to the procedures used for wild-type rubredoxin.

In both structure determinations [on RdPf (Kurihara *et al.*, 2001, 2003) and mut-RdPf (Chatake *et al.*, 2003)], the neutron data were phased by the non-hydrogen positions from the X-ray structure analysis (Bau *et al.*, 1998; Day *et al.*, 1992). Hydrogen/deuterium atoms whose positions could be predicted stereochemically (i.e., most of the H/D atoms from C-H and N-H bonds) were initially placed at calculated positions, while those whose positions could not be predicted (most H/D atoms from O-H bonds) were located from difference-Fourier maps (*vide infra*). The entire structure was eventually refined by a least-squares procedure (Brünger, 1992). Summaries of the results from the data acquisition and the structural refinement of the two structures have been published (Kurihara *et al.*, 2001, 2003; Chatake *et al.*, 2003).

3. Discussion

Before launching into a detailed discussion of the results, we should mention that both samples of rubredoxin used in this study, the wild-type protein (RdPf) as well as the triple mutant (mut-RdPf), have been partially deuterated. This was accomplished by dissolving the protein in D_2O prior to crystallization, a process that causes most of the exchangeable H atoms in the molecule (most of the hydrogens in N-H and O-H bonds) to be replaced by deuterium. This topic will be discussed at greater length later in this paper, in the section on H/D exchange.

3.1. Effects of high resolution

First and foremost, it should be pointed out that the high level of resolution of these structure determinations (the highest resolution of any single-crystal neutron analysis published to date, albeit on a relatively small protein of molecular weight, ~6 kDa) allows us to see an unprecedented level of detail. Figure 1 shows a typical example: a Fourier map near the Trp36 residue of the mut-RdPf protein. One can clearly distinguish the H atoms (negative or red contours) from the D, C and N atoms (positive or blue contours), as well as resolve the individual atoms of each C-H bond. Another indication of the high level of resolution is the fact that one can plainly see the "dimple", or hole, in the middle of the six-membered ring. Finally, it can be seen that the original N-H bond of this particular tryptophan residue is now an N-D bond (blue contours) after H/D exchange, while the C-H bonds (red contours for the H positions) have remained unchanged.

3.2. Directions of O-H bonds

Not all H positions can be predicted from an X-ray crystal structure. Once the non-hydrogen positions (those of C, N, and O atoms) have been located from an X-ray analysis of a protein, it is relatively straightforward to calculate the H positions of the C-H bonds, as

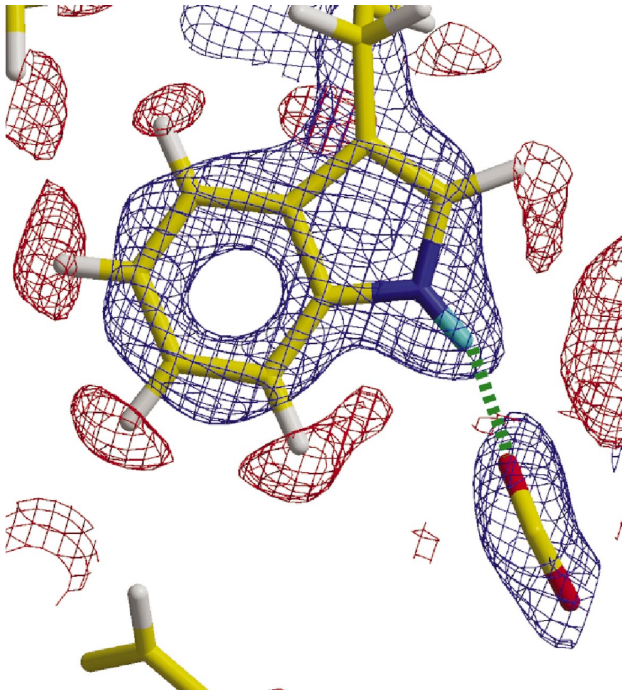

Figure 1

A neutron Fourier map of mut-RdPf near the Trp36 residue. Blue and red contours show positive and negative densities, respectively. Note the positive contours of N-D group, as opposed to the negative contours of the H atoms of the C-H bonds.

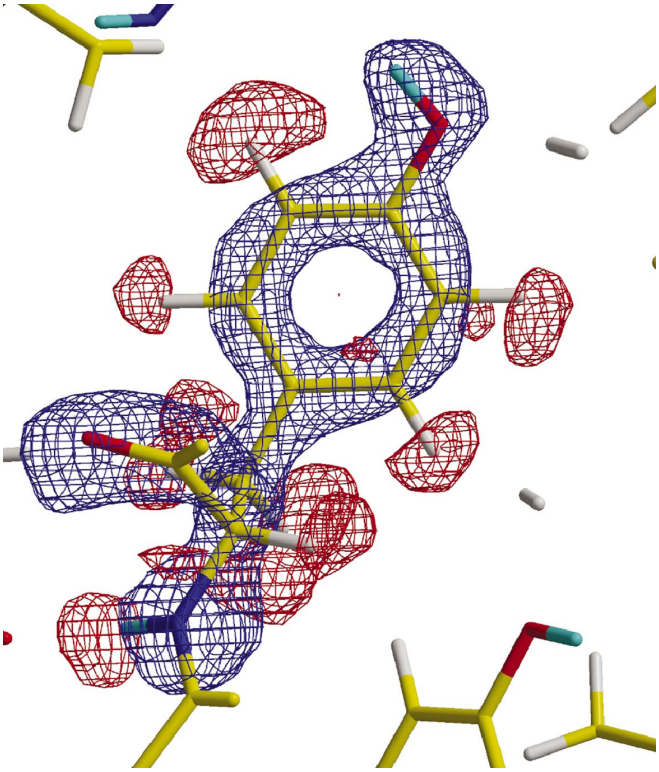

Figure 2

The Fourier map near Tyr12 of wild-type RdPf. Note that the direction of the O-H bond, which often cannot be predicted from X-ray studies, is unambiguous in this neutron map. Also note that the O-H bond has been deuterated, while the C-H bonds have not.

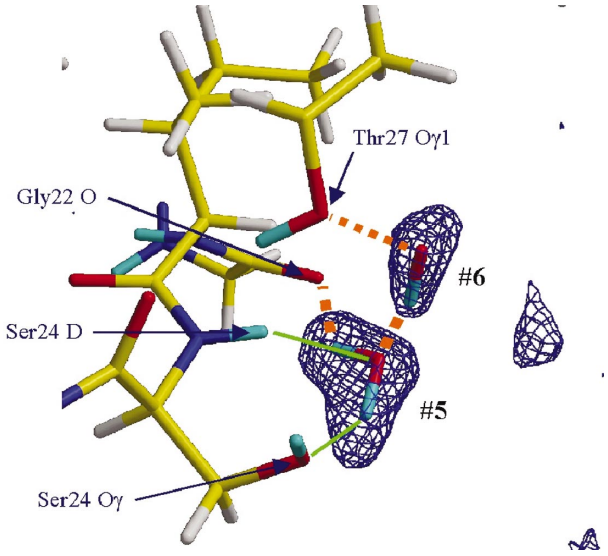

Figure 3

(F_O-F_C) omit map showing some of the hydration water molecules near residues Gly22, Ser24 and Thr27 of wild-type RdPf. Note the triangular shape of water molecule #5, showing that all three (D, O, D) are well-ordered. In contrast, the contours of water molecule #6 have an ellipsoidal shape (stick-shape), showing that only the O-D portion of it is ordered. Also note that this map allows us to unambiguously identify the H-bond donor and the H-bond acceptor of the (Ser24 O_γ)…D_2O(#5) hydrogen bond (bottommost green line; see text)

well as virtually all of the N-H bonds. Not so with O-H bonds. Because of free-rotation about the C-O bond, the hydrogen atoms have some positional ambiguity. For O-H's attached to *aliphatic* carbons (e.g. those of Ser and Thr residues), the usual assumption is that the O-H bond is in one of the three staggered positions relative to the sp^3-hybridized carbon atom, but one cannot be absolutely sure which one of those three possibilities is the correct one without actually locating the H position directly. On the other hand, for O-H groups attached to *aromatic* carbons (e.g., those of Tyr residues), one generally assumes that the O-H bond is coplanar with the aromatic ring, but once again there is a twofold uncertainty. In our case (see Figure 2, which shows the map near the Tyr12 residue of the wild-type protein) we can see the position of the O-D bond quite unambiguously.

3.3. Hydrogen-bond donors and acceptors

From an X-ray structure determination it is often not possible to identify with certainty, in a hydrogen bond, which atom is the acceptor and which is the donor. Take the map shown in Figure 3, for example. Note the region between the Ser24 O(γ) atom and water #5 (i.e. the bottommost green line). From the oxygen positions alone (red atoms) it is not possible to tell if this H-bond is of the type O_γ(Ser)–H…O(#5), or O_γ(Ser)…H–O(#5). But Figure 3 unambiguously shows that it is the latter: that is, O(#5) can be identified as the donor and O_γ(Ser) as the acceptor.

3.4. Shapes and sizes of hydration water molecules

From the shapes and sizes of various hydration molecules found in the Fourier maps, we have been able to categorize these D_2O's into three classes: (i) triangular-shaped peaks (in which all three atoms, D, O and D, are ordered); (ii) ellipsoidal-shaped contours (in

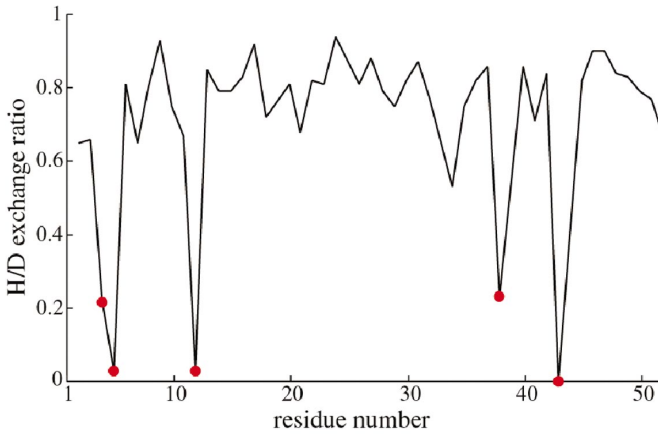

Figure 4

The deuterium populations of the main-chain amide bonds of mut-RdPf, plotted against residue number, as determined by neutron diffraction. The red circles (corresponding to residues 4, 5, 12, 38 and 43) indicate the positions of N-H bonds whose hydrogens have not engaged in H/D exchange to an appreciable extent.

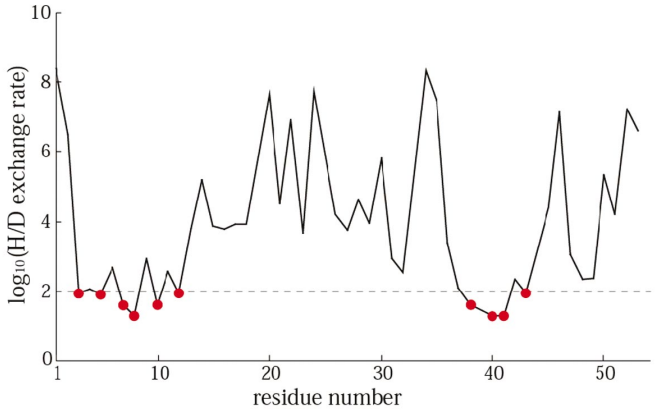

Figure 5

Results of the NMR H/D exchange experiments of RdPf (data taken from Hernandez *et al.*, 2000), showing the logarithm of exchange rates plotted against residue number. Note that the slowest-exchanging protons (red circles) are situated roughly in two regions, surrounding the Cys5/Cys8 and Cys38/Cys41 residues.

which two of the three atoms of the hydration molecule are ordered: either O,D or D,D); and (iii) spherical peaks (in which the entire D_2O molecule is rotationally disordered). In general, we have found that the best-ordered water molecules (with triangular-shaped peaks) are, as expected, those that are closest to the surface of the protein molecule. The topic of hydration is discussed in greater detail in an accompanying article (Chatake & Niimura, 2003), as well in a recent publication (Ostermann *et al.*, 2002), from members of the JAERI group.

In the earlier high-resolution X-ray study (0.95 Å) it was also possible to locate about half of the hydrogen atoms in the protein (Bau *et al.*, 1998), but very few of the H atoms in water molecules were visible in that investigation.

3.5. H/D exchange results

An important role of neutron diffraction is its ability to distinguish hydrogen from deuterium. Since these isotopes have negative and positive neutron scattering factors respectively, they effectively appear as though they were different elements in a neutron difference map (as has already been shown in Figures 1 and 2). In our case, the fact that our rubredoxin protein was dissolved in D_2O *before* crystallization means that in principle we should be able to distinguish those regions of the protein which are more "exposed" to the solvent (which presumably would be deuterated easily) from other regions which would be less prone to H/D exchange. It turns out that, of the numerous H atoms in a protein, it is the N-H hydrogens of the main-chain backbone that are most sensitive in this respect. That is because many of those N-H groups are involved in key hydrogen-bonding interactions within the protein that contribute to the structural integrity of the molecule, and thus they would be more resistant to H/D exchange. Consequently, one could get some indirect information about the unfolding patterns of a protein from its H/D exchange behavior. This field of research is being actively explored by NMR investigators, and in the case of RdPf the NMR H/D exchange behavior has been well studied (Hernandez *et al.*, 2000; Hiller *et al.*, 1997).

In a neutron diffraction study, what is done in practice is to refine the populations of the H and D atoms of the backbone N-H groups by determining their net neutron scattering amplitudes. A hydrogen atom has a neutron scattering length of –3.74 fm while the value for deuterium is +6.68 fm, and so (for example) if a certain site is 50%/50% occupied by H and D then the net scattering value at that position will have an intermediate value, or +1.47 fm. Thus, one can easily work back from the experimental scattering amplitudes to yield the corresponding H/D ratios.

Our results for rubredoxin are shown in Figure 4. We find that most of the backbone N-H groups have become deuterated, and that very few (less than half a dozen) have remained as hydrogen (red circles in Figure 4). Moreover, we find that many of the "unexchanged" hydrogen atoms are in two regions of the protein near the FeS_4 redox center of the molecule (one of these two regions is shown in Figure 6). Interestingly, the NMR results (Figure 5) also show that there are two predominant regions that have resisted deuterium incorporation, and that they are also largely centered around the FeS_4 center (Hernandez *et al.*, 2000). Thus, the two techniques (NMR and neutron diffraction) are in reasonable agreement, even though one measurement is dynamic and in solution, while the other is static and in the solid state.

3.6. Thermostability of rubredoxin from *P. furiosus*

What is the overall conclusion? Why is this particular rubredoxin, from the hyperthermophile *Pyrococcus furiosus*, so much more stable than rubredoxins from normal mesophilic (room-temperature) microorganisms? The reason, from the neutron structure, is not immediately obvious. We did not find an unusually large number of hydrogen bonds or salt bridges in this small protein. However, we did find one structural feature that might be worth mentioning, and that involves the N-terminus region of the protein. This area is rich in hydrogen-bonding interactions, which form an "arc" of H-bonds involving (i) Asp13, (ii) a D_2O molecule, (iii) Ala1, (iv) Glu14, and (v) finally ending up in Trp3 and Phe29 (Figure 7). It had been earlier postulated, from X-ray data [2,3], that residue Glu14, which is absent in mesophilic rubredoxins, appears to be extensively involved in H-bonding. The neutron structure certainly bears this out, and shows in explicit detail exactly how this set of hydrogen bonds are formed. Note, for example, that in Figure 8 one can even see the three D atoms around the N-terminus itself, showing that it is protonated (ND_3^+). It is conceivable that this

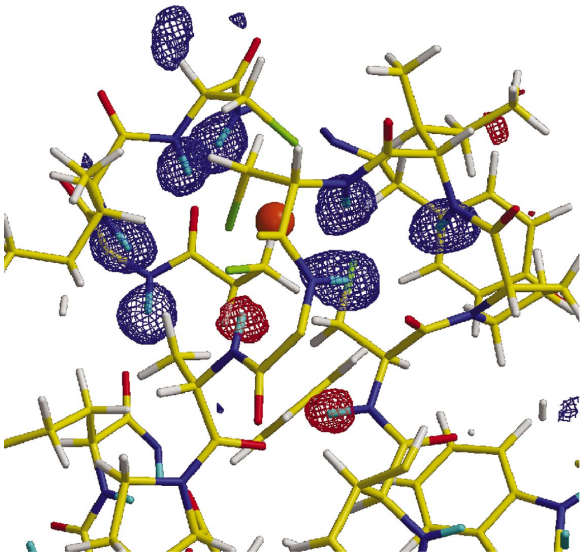

Figure 6

(F_O-F_C) omit map near the iron-sulfur cluster of RdPf. What is illustrated here are some of the hydrogen atoms (red peaks) and deuterium atoms (blue peaks) of the backbone N-H groups near the FeS_4 region. Many of the N-H groups that have not exchanged with deuterium (see red circles of Figure 4) are near the FeS_4 redox center. This plot represents one of the two regions near the FeS_4 core in which unexchanged backbone N-H bonds are found.

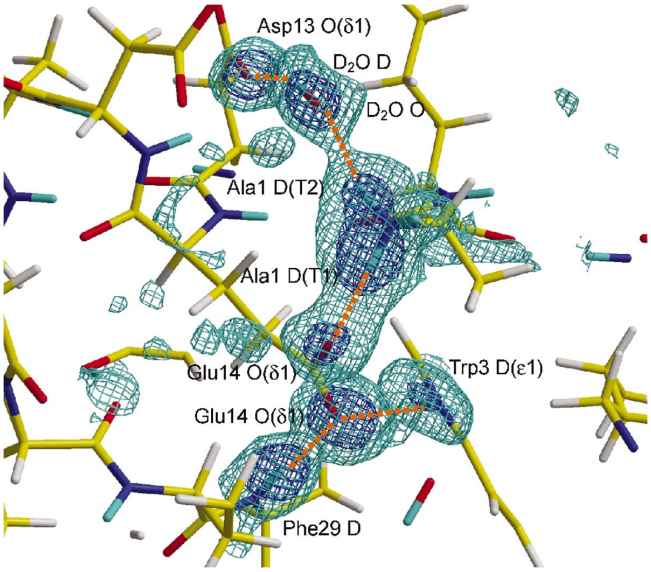

Figure 7

(F_O-F_C) omit map showing the "arc" of hydrogen bonds around the N-terminal region of wild-type rubredoxin. Contouring levels are 2.5σ (cyan) and 4.5σ (dark blue), and hydrogen bonds are shown as dotted lines.

strong network of hydrogen bonds (Figure 7) might be a contributing factor towards the thermostability of RdPf.

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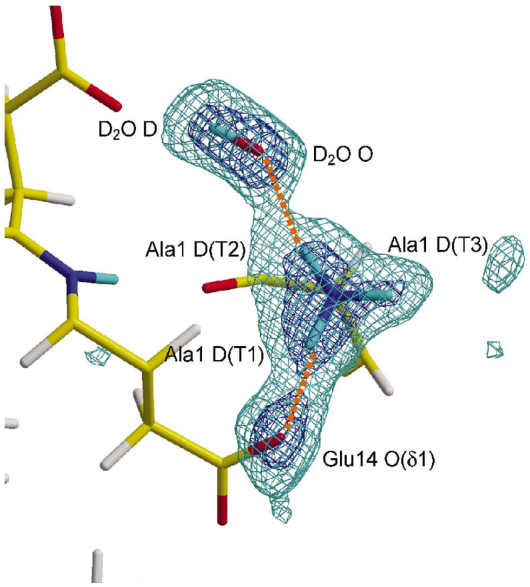

Figure 8

Close-up view of the region near the N-terminus of wild-type RdPf, clearly showing the triangular shape of the ND_3^+ group at the Ala1 terminus.

References

Bau, R.; Rees, D. C.; Kurtz, D. M.; Scott, R. A.; Huang, H. S.; Adams, M. W. W.; Eidsness, M. K. *J. Biol. Inorg. Chem.* **1998**, *3*, 484-493.
Brünger, A. T. *X-PLOR Version 3.1. A System for X-ray Crystallography and NMR*, Yale University Press, Conneticut, **1992**.
Chatake, T.; Niimura, N., *Proceedings of the ISDSB*, **2003**, (accompanying article, to be published in the *Journal of Synchrotron Research*)
Chatake, T.; Tanaka, I.; Adams, M.W.W.; Jenney, F.E.; Tsyba, I.; Bau, R.; Niimura, N., *J. Amer. Chem. Soc.*, **2003**, submitted for publication.
Day M W; Hsu B T; Joshua-Tor L; Park J B; Zhou Z H; Adams M W; Rees D C. *Protein Science*, **1992**, *1*, 1494-507.
Hernandez, G.; Jenney, F.E. Jr.; Adams, M.W.W.; Lemaster, D.M. *Proc. Nat. Acad. Sci.*, **2000**, *97*, 3166-3170.
Hiller, R.; Zhou, Z.H.; Adams, M.W.W.; Englander, S.W. *Proc. Nat. Acad. Sci.*, **1997**, *94*, 11329-11332.
Jenney, F. E., Jr.; Adams, M. W. W. *Methods Enzymol.* **2001**, *334*, 45-55
Kurihara, K.; Tanaka, I.; Adams, M.W.W.; Jenney, F.E.; Moiseeva, N.; Bau, R.; Niimura, N. *J. Phys. Soc. Japan, Suppl. A*, **2001**, *70*, 400-402.
Kurihara, K.; Tanaka, I.; Adams, M.W.W.; Jenney, F.E.; Moiseeva, N.; Bau, R.; Niimura, N., **2003**, manuscript in preparation.
Niimura, N.; Karasawa, Y.; Tanaka, I.; Miyahara, J.; Takahashi, K.; Saito, H.; Koizumi, S.; Hidaka, M. *Nucl. Instrm. Methods Phys. Res., Sect. A*, **1994**, *349*, 521-525.
Ostermann, A.; Tanaka, I.; Engler, N.; Niimura, N.; Parak, F.; Niimura, N., *Biophys. Chem.*, **2002**, *95*, 183-193.
Stetter, K.O., in "Extremophiles", Horikoshi, K.; Grant, W.D., eds.,Wiley-Liss, New York, **1998**, pp 1-24.
Tanaka, I.; Kurihara, K.; Chatake, T.; Niimura, N. *J. Appl. Crystallogr.* **2002**, *35*, 34-40;
Tanaka, I.; Niimura, N.; Mikula, P. *J. Appl. Crystallogr.* **1999**, *32*, 525-529.
Verhagen, M.F.J.M.; Menon, J.; Gerrit, J.; Adams, M. W. W. *Methods Enzymol.* **2001**, *330*, 25-30.
Zierenberg, R.A.; Adams, M.W.W. *Proc. Nat. Acad. Sci. (USA)*, **2000**, *97*, 12961-2.

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