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Production and preliminary analysis of perdeuterated yeast inorganic pyrophosphatase crystals suitable for neutron diffraction

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Yeast inorganic pyrophosphatase (Y-PPase) is a model system for studying phosphoryl-transfer reactions catalysed by multiple metal ions. To understand the process requires knowledge of the positions of the protons in the active site, which can be best achieved by neutron diffraction analysis. In order to reduce the hydrogen incoherent-scattering background and to improve the signal-to-noise ratio of the neutron reflections, deuterated protein was produced. Deuterated protein 96% enriched with deuterium was produced in high yield and crystals as large as 2 mm on one side were obtained. These crystals have unit-cell parameters a = 58.9, b = 103.9, c = 117.0 Å, $\alpha = \beta = \gamma = 90^\circ$ at 273 K and diffract neutrons to resolutions of 2.5–3 Å. The X-ray structure of the perdeuterated protein has also been refined at 273 K to 1.9 Å resolution.

1. Introduction

Metal-activated soluble inorganic pyrophosphatases (PPases) catalyse the hydrolysis of pyrophosphate to orthophosphate. These enzymes are classified into two groups, families I and II, based on sequence conservation (Shintani *et al.*, 1998; Young *et al.*, 1998). *Saccharomyces cerevisiae* PPase (Y-PPase) belongs to family I (Sivula *et al.*, 1999). Y-PPase kinetics have been extensivelly studied: for example, F^- inhibition has been investigated (Baykov *et al.*, 2000; Pohjanjoki *et al.*, 2001) and most recently the microscopic rate constants have been determined by stopped-flow and quenched-flow techniques (Halonen *et al.*, 2002). The structures of several reaction intermediates have been solved at resolutions as high as 1.15 Å (Harutyunyan *et al.*, 1996; Heikinheimo, Lehtonen *et al.*, 1996; Heikinheimo *et al.*, 2001). Knowledge of the positions of the protons in the active site is required to connect the catalytic mechanism and pH-rate profiles to the structure at the quantum-mechanical level, but this has not yet been possible despite the 1.15 Å resolution of our X-ray analysis because protons scatter X-rays very weakly.

Conversely, H atoms have a significant neutron scattering cross-section and can be readily located by neutron diffraction. However, ^{1}H is problematical in neutron scattering studies. Firstly, it has a large incoherent-scattering cross-section (80.27 barns), which increases the background and thus hides weak reflections. Secondly, it has negative coherent-scattering length (-0.374×10^{-12} cm), unlike most other atoms, which leads to non-contiguous density between ^{1}H and other protein atoms. Replacing hydrogen with deuterium avoids these problems because it has a positive coherent-scattering length (0.667×10^{-12} cm) and a relatively small incoherent-scattering cross-section (2.05 barns). The signal-to-noise ratio of neutron diffraction data is therefore significantly improved if perdeuterated protein is be produced. Because the flux of neutron sources is low and the overall scattering power of neutrons is weak, much larger crystals ($\geq$1 mm^3) are needed to obtain detectable reflections; the size needed depends on the size of the unit cell. Although a number of ^{1}H protein structures have been solved by neutron diffraction using crystals soaked in D_2O to reduce the background (Bon *et al.*, 1999; Borah *et al.*, 1985; Chatake *et al.*, 2002; Cheng & Schoenborn, 1990; Coates *et al.*, 2001; Habash *et al.*, 2000; Niimura *et al.*, 1997; Ostermann *et al.*, 2002; Wlodawer *et al.*, 1986, 1989), these proteins have had small primitive unit-cell volumes (<200 000 Å^3). The unit-cell volume of Y-PPase is over 700 000 Å^3, meaning that perdeuterated protein (where every H is replaced with D) must be produced. Shu *et al.* (1996) have reported production of perdeuterated sperm-whale myoglobin at about 50 mg protein per litre of culture and the neutron structure was solved at 2.0 Å resolution from crystals with a volume of about 2.5 mm^3 (Shu *et al.*, 2000). Perdeuterated staphylococcal nuclease (SNase) has been produced at 80 mg protein per litre of culture, but only its 1.9 Å resolution X-ray diffraction structure has been published (Gamble *et al.*, 1994).

Refining both heavy-atom (non-H) and D atoms together against neutron diffraction data alone would lead to the data-to-parameter ratio being too low and the refinement being underdetermined.

We have produced and crystallized perdeuterated PPase (D-Y-PPase) and demonstrated that the crystals are suitable for neutron analysis. In order to refine the protein structure against neutron diffraction data and to check for isotope-effect-induced changes, the X-ray structure of D-Y-PPase was determined and analysed at 1.9 Å resolution.

2. Materials and methods

2.1. Production and purification of perdeuterated Y-PPase

Perdeuterated Y-PPase was expressed in *Escherichia coli* strain XL2-blue (Stratagene, CA, USA) transformed with a plasmid containing the *PPA1* gene under the *tac* promoter (Heikinheimo, Pohjanjoki *et al.*, 1996). Cells were inoculated from H_2O LB plates into 1 ml 2×YT medium (Rodriquez & Tait, 1983) containing 10%(*v*/*v*) D_2O and left for 6.5 h in an incubator shaker at 310 K. 200 µl of cells was then used to inoculate 25 ml 2×YT medium containing 40%(*v*/*v*) D_2O and the cells were grown for 16.5 h. 1 ml of cells adapted to 40%(*v*/*v*) D_2O was used to inoculate 100 ml 2×YT medium containing 99.9%(*v*/*v*) D_2O and left to grow for 24 h. The cells were then harvested and suspended in 100 ml CELTONE-d (Spectra Stable Isotopes, MD, USA). After 1 h of adaptation, the cells were transferred to the production medium, CELTONE-d, in a total volume of 1 l. After 6 h, cells were induced with 1 m*M* isopropyl β-D-thiogalactopyranoside. For each growth, production was carried out at 310 K in four aliquots in 2 or 3 l Erlenmeyer flasks (Fig. 1).

The purification was performed in ^1H_2O using the published procedure (Heikinheimo, Pohjanjoki *et al.*, 1996). The molecular weight of hydrogenated Y-PPase is 32 180 Da and that of perdeuterated protein in ^1H_2O 33 805 Da as determined experimentally by API QSTAR Pulsar ESI mass spectrometry (Applied Biosystems, CA, USA), corresponding to a deuterization level of 95.6%.

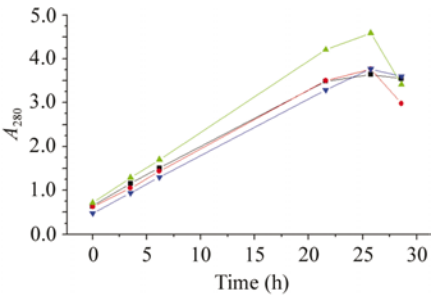

Figure 1
The growth curve of *E. coli* producing Y-PPase in CELTONE-d. The four curves are for the four Erlenmeyer flasks used in this step. (Figure generated using *Origin* v.5.0, OriginLab, MA, USA.)

2.2. Crystallization

For crystallization, we made deuterated phosphate buffer pH 6.0 from Na_2DPO_4 and KD_2PO_4 prepared by recrystallizing ^1H salts in D_2O. The other stock solutions were made by dissolving the anhydrous salts in D_2O. Crystallizations were all performed in sitting drops (Chryschem plates; Hampton Research, CA, USA). The starting drop composition was 10 mg ml^{-1} protein, 30 m*M* MES pH 6.0, 1 m*M* P_i pH 6.0, 1 m*M* $MnCl_2$ and 14%(*v*/*v*) perdeuterated methyl-2,4-pentanediol (MPD; Isotec Inc, OH, USA) in D_2O. This was equilibrated against well solution consisting of 16%(*v*/*v*) perdeuterated MPD in D_2O at 277 K. Large crystals were grown by repeated macroseeding. While crystals for neutron diffraction experiments were grown in the presence of perdeuterated MPD, the crystal used for X-ray structure determination was grown in H-MPD.

2.3. X-ray diffraction analysis

The crystals were mounted in glass capillaries and data were collected at 273 K using a Rigaku RU-200B (Rigaku International Corporation, Japan) rotating-anode X-ray source equipped with MSC confocal optics, a MAR345 image-plate detector system (X-ray Research GmbH, Germany) and an Oxford 600 cryostream (Oxford Cryosystems Ltd, UK). An entire data set was collected from a single six-month-old crystal of dimensions 0.7 × 0.3 × 0.2 mm and the data were processed with *HKL* v.1.97.2 (Otwinowski & Minor, 1997). Refinement was performed using *CNS* v.0.5 and finally *CNS* v.1.1 (Brünger *et al.*, 1998). Refinement was started using the 2.0 Å ^1H-Y-PPase product complex collected at 288 K (Heikinheimo, Lehtonen *et al.*, 1996; PDB code 1wgj). The data-collection statistics and final refinement statistics are shown in Table 1.

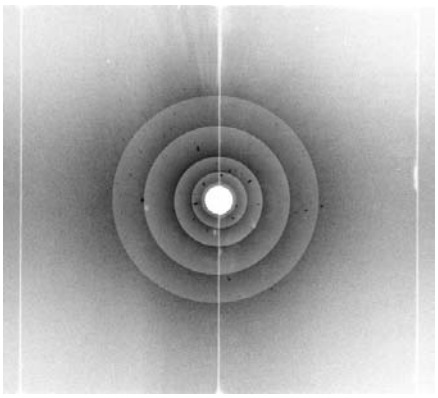

Figure 2
A Laue neutron diffraction image collected from a stationary perdeuterated Y-PPase crystal. The composite image is scaled at different contrast levels to make most of the reflections visible. (The figure was composited using *Photoshop*, Adobe Systems Incorporated, CA, USA).

Table 1
X-ray statistics.

Values in parentheses are for the last resolution shell.

Data collection	
Temperature (K)	273
No. crystals	1
$R_{merge}(I)$ (%)	6.1 (28.2)
Resolution (Å)	30.0–1.9
Independent observations	51797
Multiplicity	3.6
Completeness (%)	90.2 (84.8)
Refinement: final model	
Reflections $\|F\| > 2\sigma(F)$	46323
R_{work} (%)	13.99
R_{free} (%)	16.38
No. atoms	5035
Multiple conformations	136 (2.7%)
Waters	534
B factors (Å^2)	
Protein	21.9
Phosphate	15.7
Mn^{2+}	16.2
Waters	36.2
R.m.s.d. bonds (Å)	0.006
R.m.s.d. angles (°)	1.63
R.m.s.d. dihedrals (°)	24.8
Ramachandran plot: residues in most favoured region (%)	87.8

3. Neutron diffraction

The neutron diffraction properties of perdeuterated Y-PPase crystals were tested using the quasi-Laue (λ = 3.5 Å, dλ/λ ≃ 25%) instrument LADI at the Institute Laue Langevin (ILL), which is equipped with a cylindrical neutron image-plate detector (Myles *et al.*, 1998). This instrument has been used for a number of protein neutron diffraction experiments (*e.g.* Bon *et al.*, 1999; Coates *et al.*, 2001; Ho *et al.*, 2001; Niimura *et al.*, 1997). Crystals were mounted in quartz capillaries in a similar way to that used for X-ray data collection at 273 K. The crystals were exposed for 20 h per frame (Fig. 2). Although they diffract extremely well, PPase crystals are sensitive both to changes in temperature and to mechanical stress. A Peltier cooler was used to keep the temperature within a very narrow range close to 275 K.

4. Results and discussion

4.1. Production, purification and crystallization of perdeuterated Y-PPase

In order to minimize the costs of deuterated media, we tested expression using D9 minimal media with perdeuterated succinic acid as the sole carbon source. However, the *E. coli* grew very poorly and the D-Y-PPase yield was only about 10 mg per litre of culture. For comparison, using the same construct for the production of ^{1}H-Y-PPase usually produced 200–600 mg l^{-1} on rich 2×YT media. We therefore tested minimal media with ^{1}H glucose or ^{1}H succinic acid as carbon sources and in both cases the yield of PPase was low, suggesting that the minimal media, not the deuterated material, was slowing growth. We therefore decided to try rich perdeuterated medium, CELTONE-d, as this had been successfully used for production of SNase (Gamble *et al.*, 1994).

As reported previously (Marmur & Schildkraut, 1961), *E. coli* needs gradual adaptation to D_2O, but we also had to increase the ratio of bacteria to medium used in the last step. For ^{1}H-production, we infected new media with about 1% of the previous culture. This worked poorly for D-Y-PPase; the yield on CELTONE-d was not significantly better than with minimal medium. We therefore increased the amount of *E. coli* cells used for the final inoculation into CELTONE-d media tenfold and the yield of D-Y-PPase also increased by a factor of ten, producing 130 mg of purified perdeuterated Y-PPase per litre of culture. These yields are as high or higher than most literature values for the production of perdeuterated protein, although we note that yields of over 200 mg per litre of deuterated Ef Tu have been reported using a deuterated succinate minimal medium (Paliy *et al.*, 2003). However, this material has not been purified and the deuteration level has not been reported.

The perdeuterated Y-PPase was 95.6% deuterium-enriched at the non-exchangeable hydrogen positions as determined by mass spectrometry. This is better than expected, as about 15% of the final cell mass was grown without deuterated carbon source (see §2) and the Y-PPase expression construct is leaky and can produce a significant amount of Y-PPase before induction. This is essentially the same level of deuterization as was achieved for SNase (Gamble *et al.*, 1994).

The final dimensions of the crystals grown for neutron diffraction were about 2 × 1.5 × 1 mm. Perdeuterated Y-PPase product complex crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 58.9, b = 103.9, c = 117.0 Å at 273 K.

4.2. X-ray diffraction structure

The 273 K X-ray structure of D-Y-PPase at 1.9 Å resolution is very similar to the ^{1}H-Y-PPase 258 K structure at 2.0 Å resolution (Heikinheimo, Lehtonen *et al.*, 1996; PDB code 1wag) and the ^{1}H-Y-PPase 100 K structure at 1.15 Å (Heikinheimo *et al.*, 2001; PDB code 1e9g); the r.m.s.d. per C^α when the monomers were superimposed is only 0.15 Å. (There are two monomers per asymmetric unit in Y-PPase crystals.) This could be expected since large changes have not been reported for neutron structures of ^{1}H-proteins in D_2O. For example, for endothiapepsin the r.m.s.d. per C^α was 0.2 Å (Coates *et al.*, 2001), for ribonuclease-A the r.m.s.d. per atom for all protein atoms was 0.799 Å (Wlodawer *et al.*, 1986) and for insulin the r.m.s.d. per atom for all atoms was 0.36 Å (Wlodawer *et al.*, 1989).

4.3. Neutron diffraction

Neutron diffraction from a crystal volume of only ∼1 mm^3 gave observable reflections at better than <3 Å resolution (Fig. 2) at LADI. Compared with early results from hydrogenated protein where only 6 Å resolution reflections were observed, these experiments confirm that the use of highly deuterated protein can significantly lower the background scattering and thus improve diffraction data quality.

Unfortunately, PPase crystals are not stable above 277 K and the weak air flow in the cooler lead to the formation of a temperature gradient along the capillary, causing reflux and condensation of MPD from the mother liquor around the crystal. This first caused the crystal to slip and finally destroyed it before a full data set could be collected (which requires several weeks). We therefore plan to use flash-freezing cooling techniques to collect a full neutron data set at 100 K and to determine the structure of D-Y-PPase by neutron diffraction at the highest resolution possible.

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References

Baykov, A. A., Fabrichniy, I. P., Pohjanjoki, P., Zyryanov, A. B. & Lahti, R. (2000). *Biochemistry*, **39**, 11939–11947.
Bon, C., Lehmann, M. S. & Wilkinson, C. (1999). *Acta Cryst.* D**55**, 978–987.
Borah, B., Chen, C. W., Egan, W., Miller, M., Wlodawer, A. & Cohen, J. S. (1985). *Biochemistry*, **24**, 2058–2067.
Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Chatake, T., Kurihara, K., Tanaka, I., Adams, M. W. W., Jenney, F. E., Tsyba, I., Bau, R. & Niimura, N. (2002). *Appl. Phys. A*, **74**, S1280–S1282.
Cheng, X. & Schoenborn, B. P. (1990). *Acta Cryst.* B**46**, 195–208.
Coates, L., Erskine, P. T., Wood, S. P., Myles, D. A. A. & Cooper, J. B. (2001). *Biochemistry*, **40**, 13149–13157.
Gamble, T. R., Clauser, K. R. & Kossiakoff, A. A. (1994). *Biophys. Chem.* **53**, 15–25.
Habash, J., Raftery, J., Nuttall, R., Price, H. J., Wilkinson, C., Kalb, A. J. & Helliwell, J. R. (2000). *Acta Cryst.* D**56**, 541–550.
Halonen, P., Baykov, A. A., Goldman, A., Lahti, R. & Cooperman, B. S. (2002). *Biochemistry*, **41**, 12025–12031.
Harutyunyan, E. H., Kuranova, I. P., Vainshtein, B. K., Höhne, W. E., Lamzin, V. S., Dauter, Z., Teplyakov, A. V. & Wilson, K. S. (1996). *Eur. J. Biochem.* **239**, 220–228.
Heikinheimo, P., Lehtonen, J., Baykov, A., Cooperman, B. S., Lahti, R. & Goldman, A. (1996). *Structure*, **4**, 1491–1508.
Heikinheimo, P., Pohjanjoki, P., Helminen, A., Tasanen, M., Cooperman, B. S., Goldman, A., Baykov, A. & Lahti, R. (1996). *Eur. J. Biochem.* **239**, 138–143.
Heikinheimo, P., Tuominen, V., Ahonen, A.-K., Teplyakov, A., Cooperman, B. S., Baykov, A. A., Lahti, R. & Goldman, A. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 3121–3126.
Ho, J. X., Declercq, J. P., Myles, D. A. A., Wright, B. S., Ruble, J. R. & Carter, D. C. (2001). *J. Cryst. Growth*, **232**, 317–325.
Marmur, J. & Schildkraut, C. L. (1961). *Nature (London)*, **189**, 636–638.
Myles, D. A. A., Bon, C., Langan, P., Cipriani, F., Castagna, J. C., Lehmann, M. S. & Wilkinson, C. (1998). *Physica B*, **241–243**, 1122–1130.
Niimura, N., Minezaki, Y., Nonaka, T., Castagne, J.-C., Cipriani, F., Høghøj, P., Lehmann, M. S. & Wilkinson, C. (1997). *Nature Struct. Biol.* **4**, 909–914.
Ostermann, A., Tanaka, I., Engler, N., Niimura, N. & Parak, F. G. (2002). *Biophys. Chem.* **95**, 183–193.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Paliy, O., Bloor, D., Brockwell, D., Gilbert, P. & Barber, J. (2003). *J. Appl. Microbiol.* **94**, 580–586.
Pohjanjoki, P., Fabrichniy, I. P., Kasho, V. N., Cooperman, B. S., Goldman, A., Baykov, A. A. & Lahti, R. (2001). *J. Biol. Chem.* **276**, 434–441.
Rodriquez, R. L. & Tait, R. C. (1983). *Recombinant DNA Techniques: An Introduction*, p. 149. Reading, MA, USA: Addison–Wesley.
Shintani, T., Uchiumi, T., Yonezawa, T., Salminen, A., Baykov, A. A., Lahti, R. & Hachimori, A.

(1998). *FEBS Lett.* **439**, 263–267.
Shu, F., Ramakrishnan, V. & Schoenborn, B. P. (1996). *Basic Life Sci.* **64**, 309–323.
Shu, F., Ramakrishnan, V. & Schoenborn, B. P. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 3872–3877.
Sivula, T., Salminen, A., Parfenyev, A. N., Pohjanjoki, P., Goldman, A., Cooperman, B. S., Baykov, A. A. & Lahti, R. (1999). *FEBS Lett.* **454**, 75–80.
Wlodawer, A., Borkakoti, N., Moss, D. S. & Howlin, B. (1986). *Acta Cryst.* B**42**, 379–387.
Wlodawer, A., Savage, H. & Dodson, G. (1989). *Acta Cryst.* B**45**, 99–107.
Young, T. W., Kuhn, N. J., Wadeson, A., Ward, S., Burges, D. & Cooke, G. D. (1998). *Microbiology*, **144**, 2563–2571.