Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

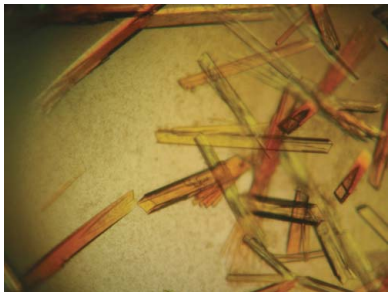
Crystallization and preliminary X-ray analysis of AzoR (azoreductase) from *Escherichia coli*

Kosuke Ito,[a] Masayuki Nakanishi,[b] Woo-Cheol Lee,[a] Hiroshi Sasaki,[a] Shuhei Zenno,[c] Kaoru Saigo,[c] Yukio Kitade[b] and Masaru Tanokura[a]*

[a]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, [b]Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, and [c]Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Correspondence e-mail: amtanok@mail.ecc.u-tokyo.ac.jp

Received 11 September 2004
Accepted 12 March 2005
Online 24 March 2005



AzoR (azoreductase), an FMN-dependent NADH-azo compound oxidoreductase from *Escherichia coli*, has been crystallized in the presence of FMN by the sitting-drop vapour-diffusion method using 2-propanol as a precipitant. AzoR catalyzes the reductive cleavage of azo groups. The crystals were found to diffract X-rays to beyond 1.8 Å resolution using a synchrotron-radiation source. The crystals belonged to the tetragonal space group $P4_22_12$, with unit-cell parameters $a = b = 92.2$, $c = 51.9$ Å. The crystals are expected to contain one subunit of the homodimer in the asymmetric unit ($V_M = 2.6$ Å^3 Da^{-1}) and to have a solvent content of 51.6%. Data sets were also collected from heavy-atom derivatives for use in phasing. As a result, crystals soaked in a solution containing K_2PtCl_4 for 23 d were found to be reasonably isomorphous to the native crystals and the presence of Pt atoms could be confirmed. The data sets from the native crystals and the K_2PtCl_4-derivatized crystals are being evaluated for use in structure determination by single isomorphous replacement with anomalous scattering.

1. Introduction

Azo dyes, synthetic organic colorants, are extensively used in printing, in food, for clinical purposes and in the cosmetics industry because of their chemical stability and ease of synthesis and utility (Meyer, 1981). Most azo dyes are released into the environment in waste water. They are not completely degraded even after wastewater treatment based on chemical procedures, which are expensive methods and often yield hazardous byproducts. In addition, the toxic and mutagenic properties of some azo dyes (Holme, 1984) has led to a demand for efficient technology for their degradation. Biological degradation using microorganisms is a useful method for decomposing the dyes under mild conditions without the problems mentioned above (Robinson *et al.*, 2001; Stolz, 2001). Therefore, a detailed understanding of the mechanisms of dye-degrading enzymes will facilitate the development of biodegradation systems.

AzoR, an enzyme catalyzing the reductive cleavage of azo groups (—N═N—), was purified from *Escherichia coli* (Nakanishi *et al.*, 2001) in order to determine the molecular basis of the biodegradation method. Biochemical studies revealed that AzoR exists as a homodimer composed of 23 kDa (200 amino-acid) subunits, as shown by gel filtration. AzoR utilizes NADH but not NADPH as an electron donor and binds FMN as a flavin cofactor. The reaction follows a ping-pong mechanism requiring 2 mol NADH to reduce 1 mol methyl red (4′-dimethylaminoazobenzene-2-carboxylic acid), a typical azo dye, to 2-aminobenzoic acid and *N*,*N*′-dimethyl-*p*-phenylenediamine. However, details of the molecular mechanism of catalysis remain unknown.

Many types of bacterial cytoplasmic azoreductases have been isolated and characterized for the purpose of environmental biotechnology (Ghosh *et al.*, 1992, 1993; Maier *et al.*, 2004; Moutaouakkil *et al.*, 2003; Rafii & Cerniglia, 1993; Ramalho *et al.*, 2004; Suzuki *et al.*, 2001; Zimmermann *et al.*, 1982). However, AzoR is different from other azoreductases reported thus far with respect to

its substrate specificity, requirement for a flavin cofactor and type of electron donor. For example, the azoreductase purified from *Bacillus* sp. by Suzuki and coworkers degrades azo compounds utilizing NADPH, but NADPH is ineffective as an electron donor for AzoR. The azoreductase purified from *Pseudomonas* KF46 by Zimmermann and coworkers utilizes FAD as a flavin cofactor; however, AzoR utilizes FMN as a flavin cofactor. Although the physiological function of AzoR remains unknown, its importance has been deduced from the wide distribution of AzoR orthologues in bacteria, as has been revealed by genome projects, such as those on *Bacillus subtilis* (Kunst *et al.*, 1997), *Streptomyces coelicolor* (Bentley *et al.*, 2002), *Salmonella typhimurium* (McClelland *et al.*, 2001), *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Pseudomonas aeruginosa* (Stover *et al.*, 2000). On the other hand, AzoR shows a moderate sequence homology in the active site to NQO1, originally called DT-diaphorase [NAD(P)H:quinone reductase; EC 1.6.99.2], a mammalian FAD-containing protein that also catalyzes the reduction of azo compounds (Bayney *et al.*, 1987). NQO1 plays an important role in detoxification and has a protective effect against mutagenicity, carcinogenicity and other toxicities by utilizing its reduction activity (Benson *et al.*, 1980; Chesis *et al.*, 1984; Smith, 1999). These facts imply that AzoR may play an important role in detoxification in bacteria.

AzoR is representative of a poorly characterized family of azo-dye reductases. Structure determination of AzoR is a first step toward the elucidation of their molecular mechanism of function. Here, we report the crystallization and preliminary X-ray crystallographic analysis and the search for heavy-atom derivatives for use in phasing. This is the first report of the crystallization of an FMN-dependent NADH-azo reductase.

2. Crystallization

The overexpression of AzoR in *E. coli* and its subsequent purification were carried out using previously described methods (Nakanishi *et al.*, 2001). The estimated pI using the program *GENETYX* (Hiller *et al.*, 2003) is 4.92. Initial crystallization trials were performed by the hanging-drop vapour-diffusion method using the crystallization kits Crystal Screens 1 and 2 (Hampton Research). Two protein solutions were used: a solution containing 23 mg ml^{-1} (0.25 m*M*) AzoR in 10 m*M* Tris–HCl pH 8.0 and a solution additionally containing 1 m*M* FMN. Each drop, consisting of 1 µl protein solution and an equal volume of reservoir solution, was equilibrated against 500 µl reservoir solution at both 278 and 293 K. The solubility of the protein in the buffers containing and not containing FMN was almost the same. Several microcrystals and two morphologically different crystals were observed in a week. Crystals of form I grew as bar-shaped crystals from condition No. 12 of Crystal Screen I [200 m*M* $MgCl_2$, 100 m*M* HEPES buffer pH 7.5 and 30%(*v*/*v*) 2-propanol as a precipitant] at 293 K. Crystals of form II grew as a cluster of thin plates from condition No. 23 of Crystal Screen I [200 m*M* $MgCl_2$, 100 m*M* HEPES buffer pH 7.5 and 30%(*v*/*v*) PEG 400 as a precipitant] at 278 K.

Trials to improve the crystallization conditions were performed for the form I and II crystals by varying the pH, protein concentration, precipitant concentration, FMN concentration and incubation temperature. Simultaneously, we also examined additives using Additive Screens 1, 2 and 3 (Hampton Research). After refinement of the crystallization conditions, form I crystals suitable for X-ray analysis were obtained from a drop made by mixing 15 µl of a solution containing 23 mg ml^{-1} protein in 10 m*M* Tris–HCl pH 8.0, 1 m*M* FMN and an equal volume of reservoir solution containing 200 m*M* $MgCl_2$, 30%(*v*/*v*) 2-propanol and 100 m*M* HEPES buffer pH 7.5. The drop was equilibrated over 500 µl reservoir solution by the sitting-drop vapour-diffusion method for one week. Clustering was reduced by lowering the temperature to 288 K; the crystal quality was maintained. Fig. 1 shows typical crystals. On the other hand, the form II crystals could not be improved to a level suitable for X-ray analysis. During optimization trials, it became clear that the drop size was a critical parameter in the size of form I crystals.

Interestingly, all the crystals that appeared in the initial crystallization trials were only found under conditions containing FMN. Not even microcrystals were found under conditions without FMN. All the crystals were yellow in colour and this colour was not lost on transferring the crystals to a reservoir solution without FMN. These results suggest that FMN is tightly bound to the protein moiety and this interaction is essential for the crystallization of AzoR.

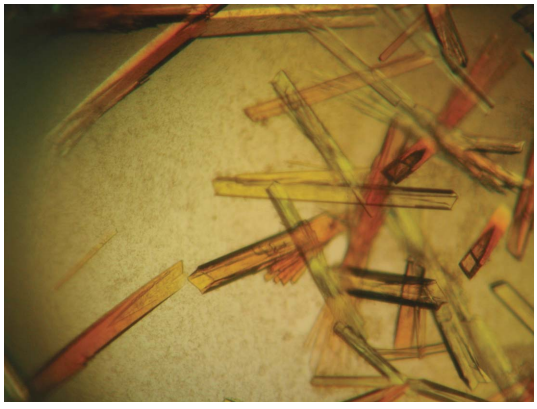


Figure 1
Crystals of AzoR from *E. coli* obtained by means of the sitting-drop vapour-diffusion method. The average dimensions of these crystals were 0.03 × 0.03 × 0.6 mm.

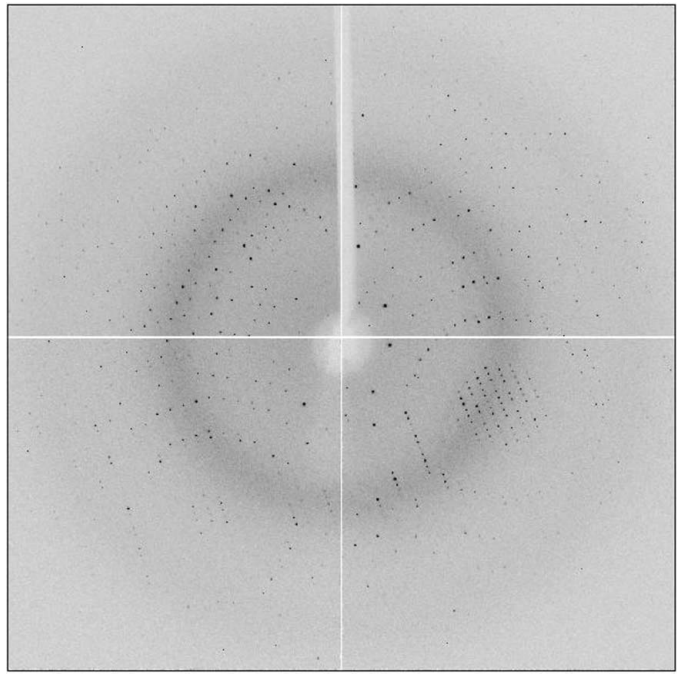


Figure 2
X-ray diffraction image of an AzoR native crystal. The edge of the detector corresponds to a resolution of 1.8 Å.

3. Data collection and processing

3.1. Native data

Form I crystals were mounted in a nylon loop (Hampton Research) and transferred to a reservoir solution containing 30%(*v*/*v*) ethylene glycol as a cryoprotectant. The crystals were flash-cooled in a stream of nitrogen using a Rigaku cryostat. Diffraction data were collected at 100 K at the BL6A station of the Photon Factory (Tsukuba, Japan) using an ADSC Quantum 4 CCD detector. The wavelength was set to 1.000 Å, the oscillation angle was set to 0.5° and the distance between the crystal and the detector was set to 150 mm. The exposure time was set to 60 s, taking into account the dynamic range of the CCD detector. The crystals diffracted X-rays to beyond 1.8 Å resolution (Fig. 2). *MOSFLM*, *SCALA* and *TRUNCATE* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) were used for processing, reduction and scaling of the diffraction data. The crystals were found to belong to the tetragonal space group $P4_22_12$, with unit-cell parameters $a = b = 92.2$, $c = 51.9$ Å. The crystals are expected to contain one molecule in the asymmetric unit and to have a solvent content of 51.6% (V_M = 2.6 Å^3 Da^{-1}; Matthews, 1968). Data-collection statistics are summarized in Table 1.

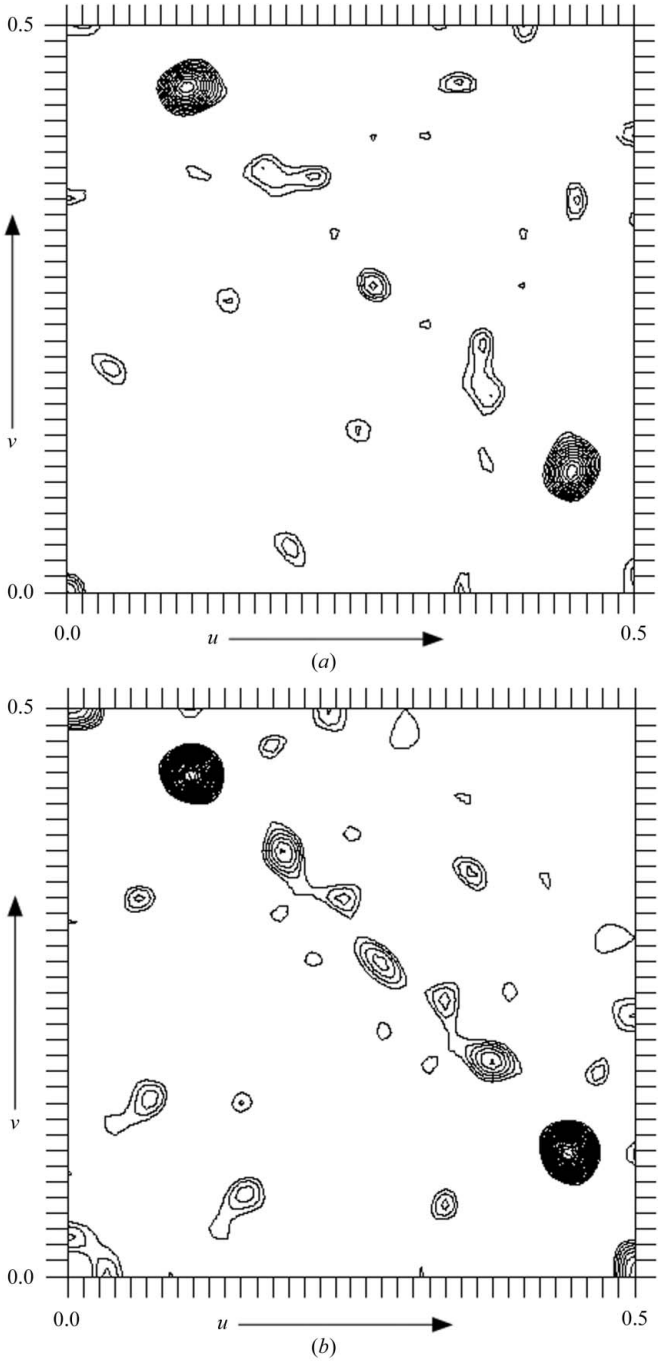


Figure 3
The Harker sections of the isomorphous difference Patterson map (*a*) and the anomalous difference Patterson map (*b*) computed with the data between 15.0 and 4.0 Å. The $w = 0.5$ section of the maps contoured at 3.0σ levels is shown.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data set	Native	K_2PtCl_4
Beamline	PF-BL6A	SPring-8 BL41XU
Wavelength (Å)	1.000	1.000
Space group	$P4_22_12$	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 92.2$, $c = 51.9$	$a = b = 92.4$, $c = 51.7$
Resolution range (Å)	65.2–1.8 (1.9–1.8)	65.3–2.5 (2.6–2.5)
Total oscillation range† (°)	191	170
Average mosaicity	0.48	0.59
No. of measured reflections	287719	106879
No. of unique reflections	21147	8145
Completeness (%)	99.7 (98.2)	99.7 (100.0)
Redundancy	13.6 (8.3)	13.1 (13.5)
Average $I/\sigma(I)$	7.0 (3.0)	11.4 (8.6)
R_{merge}‡ (%)	0.063 (0.225)	0.047 (0.078)
R_{anom}§ (%)	—	0.021 (0.025)
Scaling R factor¶ (%)	—	22.6 (20.0–2.5 Å)

† The oscillation angle was set to 0.5 and 1.0° for the native and for the K_2PtCl_4 derivative, respectively. ‡ R_{merge} for the native data is as follows: $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average. R_{merge} for the K_2PtCl_4 derivative data is defined in the same way as R_{merge} for the native data, except that the summation is executed for separated Bijvoet pairs individually. § $R_{anom} = \sum_{hkl} |\langle I^+(hkl)\rangle - \langle I^-(hkl)\rangle| / |\langle I^+(hkl)\rangle + \langle I^-(hkl)\rangle|$, where $\langle I^+(hkl)\rangle$ and $\langle I^-(hkl)\rangle$ are the averages of $I^+(hkl)$ and $I^-(hkl)$, respectively. ¶ Scaling R factor $(R_{iso}) = \sum_{hkl} \big|F_{deriv}(hkl)| - |F_{native}(hkl)\big| / \sum_{hkl} |F_{native}(hkl)|$.

3.2. Heavy-atom derivatives

Conditions for heavy-atom derivatization were screened using $KAuCl_4$, $KAu(CN)_2$, K_2PtCl_4, K_2PtCl_6, $K_2Pt(CN)_4$, *t*-$PtCl_2(NH_3)_2$, $AgNO_3$ and $KHgI_4$. Reservoir solutions saturated with each of the heavy-atom reagents were diluted 1:2, 1:4 and 1:20 with reservoir solution. The native crystals were soaked for 3, 6 and 23 d in a drop prepared by mixing 1 µl of each of the solutions containing heavy-atom compounds and 10 µl of the reservoir solution at 288 K. Diffraction data sets were collected from the crystals obtained from the drops containing the highest concentration of each heavy-atom compound and diffracted X-rays beyond 3.0 Å resolution. The derivatives were flash-cooled by the same method as described in the native crystal data collection. Diffraction data were collected at 100 K using an ADSC Quantum 4 CCD detector at the BL6A and BL18B stations of the Photon Factory and a MAR Research 165 mm CCD detector at BL41XU of SPring-8 (Harima, Japan) for the crystals soaked in the solutions for the three soaking periods. The camera distance was set to 150, 180 and 130 mm at the BL6A and BL18B stations of the Photon Factory and BL41XU of SPring-8, respectively. The exposure time was set to 60–90 s at Photon Factory and 3 s at BL41XU of SPring-8, taking into account the dynamic range of each CCD detector. A wavelength of 1.000 Å was used in order to obtain an anomalous contribution from all the heavy-atom elements except

Ag in $AgNO_3$. Programs from *CCP*4 were used to process the derivative data sets and to evaluate the presence of heavy-atom elements. Isomorphous difference Patterson maps were calculated using data in the resolution range 15.0–4.0 Å with *CNS* (Brünger *et al.*, 1998). As a consequence, the presence of a heavy-atom element was found in the crystal soaked in the K_2PtCl_4-saturated solution for 23 d by analysis of the Harker section (Fig. 3). No other candidates could be found. The K_2PtCl_4 derivative diffracted X-rays to beyond 2.5 Å resolution. To confirm the presence of platinum, an anomalous difference Patterson map was calculated and the Harker section also showed two strong peaks, suggesting the presence of one high-occupancy platinum site per asymmetric unit. These peak positions are consistent with that of the isomorphous difference Patterson map (Fig. 3). The anomalous contribution from the Pt atoms is likely to allow structure determination. Furthermore, the scaling of the derivative to the native data using *SCALEIT* showed that the K_2PtCl_4 derivative was isomorphous to the native crystals, as reflected in the unit-cell parameters $a = b = 92.2 + 0.2$ Å, $c = 51.9 - 0.2$ Å, with a relatively low scaling R factor (Table1).

4. Conclusion

Diffraction data were obtained from native crystals and a K_2PtCl_4 derivative of AzoR. Preliminary runs of *SOLVE* (Terwilliger, 2002) suggest that the K_2PtCl_4 derivative contains one Pt atom per asymmetric unit (Z score = 5.11, figure of merit = 0.30). The Pt-atom position is consistent with the strong peak position in the isomorphous and anomalous difference Patterson maps. Structure determination by single isomorphous replacement with anomalous scattering is currently in progress. In addition, the crystallization of AzoR with substrates is also under way.

We thank Dr M. Murphy of The University of British Columbia for reading the manuscript and his helpful advice. The synchrotron-radiation experiments were performed at BL6A and BL18B at the Photon Factory (Tsukuba, Japan) and BL41XU in SPring-8 (Harima, Japan) with the approval of the Photon Factory, KEK (Proposal No. 2000G307) and The Japan Synchrotron Radiation Research Institute (Proposal No. 2002A0741-RL1-np), respectively. This work was supported in part by the National Project on Protein Structural and Functional Analyses of the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Bayney, R. M., Rodkey, J. A., Bennett, C. D., Lu, A. Y. & Pickett, C. B. (1987). *J. Biol. Chem.* **262**, 572–575.
Benson, A. M., Hunkeler, M. J. & Talalay, P. (1980). *Proc. Natl Acad. Sci. USA*, **77**, 5216–5220.
Bentley, S. D. *et al.* (2002). *Nature (London)*, **417**, 141–147.
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.
Chesis, P. L., Levin, D. E., Smith, M. T., Ernster, L. & Ames, B. N. (1984). *Proc. Natl Acad. Sci. USA*, **81**, 1696–1700.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A. & Merrick, J. M. (1995). *Science*, **269**, 496–512.
Ghosh, D. K., Ghosh, S., Sadhukhan, P., Mandal, A. & Chaudhuri, J. (1993). *Indian J. Exp. Biol.* **31**, 951–954.
Ghosh, D. K., Mandal, A. & Chaudhuri, J. (1992). *FEMS Microbiol. Lett.* **77**, 229–233.
Hiller, K., Schobert, M., Hundertmark, C., Jahn, D. & Munch, R. (2003). *Nucleic Acids Res.* **31**, 3862–3865.
Holme, I. (1984). *Ecological Aspects of Color Chemistry: Developments in the Chemistry and Technology of Organic Dyes*, edited by J. Griffiths, pp. 111–128. Oxford: Society for Chemical Industry.
Kunst, F. *et al.* (1997). *Nature (London)*, **390**, 249–256.
McClelland, M. *et al.* (2001). *Nature (London)*, **413**, 852–856.
Maier, J., Kandelbauer, A., Erlacher, A., Cavaco-Paulo, A. & Gubitz, G. M. (2004). *Appl. Environ. Microbiol.* **70**, 837–844.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Meyer, U. (1981). *FEMS Symp.* **12**, 371–385.
Moutaouakkil, A., Zeroual, Y., Zohra Dzayri, F., Talbi, M., Lee, K. & Blaghen, M. (2003). *Arch. Biochem. Biophys.* **413**, 139–146.
Nakanishi, M., Yatome, C., Ishida, N. & Kitade, Y. (2001). *J. Biol. Chem.* **276**, 46394–46399.
Ramalho, P. A., Cardoso, M. H., Cavaco-Paulo, A. & Ramalho, M. T. (2004). *Appl. Environ. Microbiol.* **70**, 2279–2288.
Rafii, F. & Cerniglia, C. E. (1993). *Appl. Environ. Microbiol.* **59**, 1731–1734.
Robinson, T., McMullan, G., Marchant, R. & Nigam, P. (2001). *Bioresource Technol.* **77**, 247–255.
Smith, M. T. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 7624–7626.
Stolz, A. (2001). *Appl. Microbiol. Biotechnol.* **56**, 69–80.
Stover, C. K. *et al.* (2000). *Nature (London)*, **406**, 959–964.
Suzuki, Y., Yoda, T., Ruhul, A. & Sugiura, W. (2001). *J. Biol. Chem.* **276**, 9059–9065.
Terwilliger, T. C. (2002). *Acta Cryst.* D**58**, 1937–1940.
Zimmermann, T., Kulla, H. G. & Leisinger, T. (1982). *Eur. J. Biochem.* **129**, 197-203.