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

Crystallization and preliminary X-ray analysis of molecular chaperone-like diol dehydratase-reactivating factor in ADP-bound and nucleotide-free forms

Koichi Mori,[a] Naoki Hieda,[a] Mamoru Yamanishi,[a] Naoki Shibata[b] and Tetsuo Toraya[a]*

[a]Graduate School of Natural Science and Technology, Okayama University, Tsushima-Naka, Okayama 700-8530, Japan, and [b]Graduate School of Life Science, University of Hyogo, 3-2-1 Kouto, Ako-gun, Hyogo 678-1297, Japan

Correspondence e-mail: toraya@cc.okayama-u.ac.jp

Received 23 April 2005
Accepted 18 May 2005
Online 1 June 2005

Adenosylcobalamin (coenzyme B_{12}) dependent diol dehydratase (EC 4.2.1.28) catalyzes the conversion of 1,2-diols and glycerol to the corresponding aldehydes. It undergoes mechanism-based inactivation by glycerol. The diol dehydratase-reactivating factor (DDR) reactivates the inactivated holoenzymes in the presence of adenosylcobalamin, ATP and Mg^{2+} by mediating the release of a damaged cofactor. This molecular chaperone-like factor was overexpressed in *Escherichia coli*, purified and crystallized in the ADP-bound and nucleotide-free forms by the sandwich-drop vapour-diffusion method. The crystals of the ADP-bound form belong to the orthorhombic system, with space group $P2_12_12_1$ and unit-cell parameters a = 83.26, b = 84.60, c = 280.09 Å, and diffract to 2.0 Å. In the absence of nucleotide, DDR crystals were orthorhombic, with space group $P2_12_12_1$ and unit-cell parameters a = 81.92, b = 85.37, c = 296.99 Å and diffract to 3.0 Å. Crystals of both forms were suitable for structural analysis.

1. Introduction

Coenzyme B_{12} or adenosylcobalamin (AdoCbl) serves as a cofactor for enzymatic radical reactions (Toraya, 2003). Diol dehydratase (EC 4.2.1.28) catalyzes the coenzyme B_{12}-dependent conversion of 1,2-propanediol, 1,2-ethanediol and glycerol to the corresponding aldehydes (Lee & Abeles, 1963; Toraya *et al.*, 1976). Diol dehydratase is induced when bacteria are grown anaerobically in 1,2-propanediol-containing media (Toraya & Fukui, 1977; Forage & Foster, 1979; Toraya *et al.*, 1980). Its metabolic role is to produce both an electron acceptor and a metabolic intermediate for incomplete oxidation of 1,2-diols (Toraya *et al.*, 1979; Toraya & Fukui, 1982).

AdoCbl-dependent enzymes form an adenosyl radical and Co^{II} by homolytic cleavage of the Co—C bond of the coenzyme. Since radical enzymes form a highly reactive radical in the active site, they tend to undergo mechanism-based inactivation (suicide inactivation) or O_2 inactivation in the absence of substrate (Toraya, 2003). The dehydration of 1,2-propanediol by diol dehydratase proceeds linearly with time, but the enzymes undergo irreversible inactivation during catalysis by glycerol (Toraya *et al.*, 1976; Bachovchin *et al.*, 1977). This inactivation is a type of mechanism-based inactivation and is accompanied by irreversible cleavage of the Co—C bond of the enzyme-bound coenzyme. Since the modified cofactor remains tightly bound to the apoenzyme, this results in the inactivation of the enzyme. This inactivation seemed enigmatic because glycerol is a growth substrate for the bacteria that produce this enzyme. Rapid reactivation takes place *in situ* (in toluenized cells) when ATP and Mg^{2+} are added to the completely inactivated system in the presence of AdoCbl (Honda *et al.*, 1980; Ushio *et al.*, 1982).

We identified two open reading frames in the 3′-flanking region of the diol dehydratase genes (*pddABC*) of *Klebsiella oxytoca* as the genes encoding a diol dehydratase-reactivating factor (DDR) and designated *ddrAB* (corresponding to *pduGH*) (Mori *et al.*, 1997). Recombinant DdrA (α-subunit) and DdrB (β-subunit) form a tight $\alpha_2\beta_2$ complex that actually functions *in vitro* as a reactivating factor for glycerol-inactivated holodiol dehydratase in the presence of AdoCbl, ATP and Mg^{2+} (Toraya & Mori, 1999). O_2-inactivated holoenzyme and the inactive enzyme–cyanocobalamin complex also undergo rapid reactivation and activation, respectively, by the factor

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

	ADP-bound form	Nucleotide-free form
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 83.26, b = 84.60, c = 280.09	a = 81.92, b = 85.37, c = 296.99
Wavelength (Å)	1.00000	1.00000
Resolution range (Å)	50–2.00 (2.07–2.00)	50–3.00 (3.11–3.00)
Measured reflections	925529	79068
Unique reflections	133910	41979
Completeness (%)	99.9 (99.8)	97.7 (93.3)
$R_{\rm merge}$ (%)	8.7 (59.8)	7.9 (60.6)
Multiplicity	6.9 (5.0)	6.8 (6.7)
$I/\sigma(I)$	11.2	8.9

under the same conditions. The reactivating factor mediates ATP-dependent exchange of the enzyme-bound cyanocobalamin for free adenine-containing cobalamin. It was demonstrated that the function of the reactivating factor is to release a tightly bound adenine-lacking cobalamin from the enzyme, forming apoenzyme that is reconstitutable into active holoenzyme (Mori & Toraya, 1999). Adenine-containing cobalamins such as AdoCbl and adeninylpentylcobalamin are not released from the enzyme. It was established that the reactivation of the inactivated holoenzyme by DDR takes place in two steps: ADP-dependent cobalamin release and ATP-dependent dissociation of the apoenzyme–DDR complex. ATP plays dual roles as a precursor of ADP in the first step and as an effector to change the conformation of the factor into the low-affinity form for diol dehydratase. As suggested by fragmentary sequence homologies (Mori *et al.*, 1997), *gdrAB* (*dhaB4* and orf2b) of *Klebsiella pneumoniae* (Tobimatsu *et al.*, 1999) and *dhaFG* of *Citrobacter freundii* (Seifert *et al.*, 2001), the orthologues of *gdrAB*, were identified as the genes encoding a glycerol dehydratase-reactivating factor (GDR). It was shown that GDR reactivates the inactivated glycerol dehydratase by a similar mechanism to that of DDR (Kajiura *et al.*, 2001). Very recently, *Escherichia coli eutA* was identified as the gene encoding an ethanolamine ammonia lyase-reactivating factor (Mori *et al.*, 2004).

In this communication, crystallization and preliminary X-ray analyses of ADP-bound and nucleotide-free forms of DDR of *K. oxytoca* are reported. A paper has appeared which reported the X-ray structure of the nucleotide-free form of GDR (Liao *et al.*, 2003).

2. Materials and methods

2.1. Expression and purification of DDR

Wild-type DDR of *K. oxytoca* was purified to homogeneity from methionine-auxotrophic *E. coli* B834 (Novagen) harbouring expression plasmid pUSI2ENd(6/5b) (Toraya & Mori, 1999) grown in the presence of methionine. Cells were aerobically grown overnight at 303 K in 40 ml LB medium containing ampicillin (50 µg ml^{-1}), harvested by centrifugation, washed twice with 30 ml minimal medium (6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl per litre) and suspended in 20 ml minimal medium. The cell suspension (4 ml) was inoculated into 800 ml fresh minimal medium supplemented with 0.1 g L-lysine hydrochloride, 0.1 g L-threonine, 0.1 g L-phenylalanine, 0.05 g L-leucine, 0.05 g L-isoleucine, 0.05 g L-valine, 0.05 g L-methionine, 0.49 g $MgSO_4{\cdot}7H_2O$, 0.011 g $CaCl_2$, 4 g D-glucose and 0.05 g ampicillin per litre. After cells had been aerobically grown at 303 K for 6 h, isopropyl-1-thio-β-D-galactopyranoside was added to a concentration of 1 m*M* for induction. Cells were then aerobically grown at 303 K to the late logarithmic phase, harvested by centrifugation and washed twice with 50 m*M* potassium phosphate buffer pH 8.0. DDR was purified according to the method described previously (Toraya & Mori, 1999).

2.2. Crystallization

DDR was crystallized in nucleotide-free and ADP-bound forms under similar conditions. Purified DDR (20–40 mg ml^{-1} in 10 m*M* potassium phosphate buffer pH 8.0) was incubated at 303 K for 30 min with either 50 m*M* 2-mercaptoethanol (for the ADP-bound form) or 10 m*M* dithiothreitol (for the nucleotide-free form). 2 m*M* ADP and 2 m*M* $MgCl_2$ were added to 2-mercaptoethanol-treated DDR and the mixtures were incubated at 293 K for 20 min. Crystals were grown by the sandwich-drop vapour-diffusion method at 277 K against 0.5 ml reservoir containing 15% PEG 6000, 90 m*M* ammonium sulfate, 12 m*M* Tris–HCl pH 8.0, 20% PEG 400 and either 50 m*M* 2-mercaptoethanol (for the ADP-bound form) or 10 m*M* dithiothreitol (for the nucleotide-free form). The crystal-growth droplet was composed of 30 µl DDR solution and 30 µl reservoir solution.

2.3. Data collection

All X-ray diffraction data sets were collected at 100 K at the BL41XU beamline, SPring-8, Japan. A total of 180 images with 1°

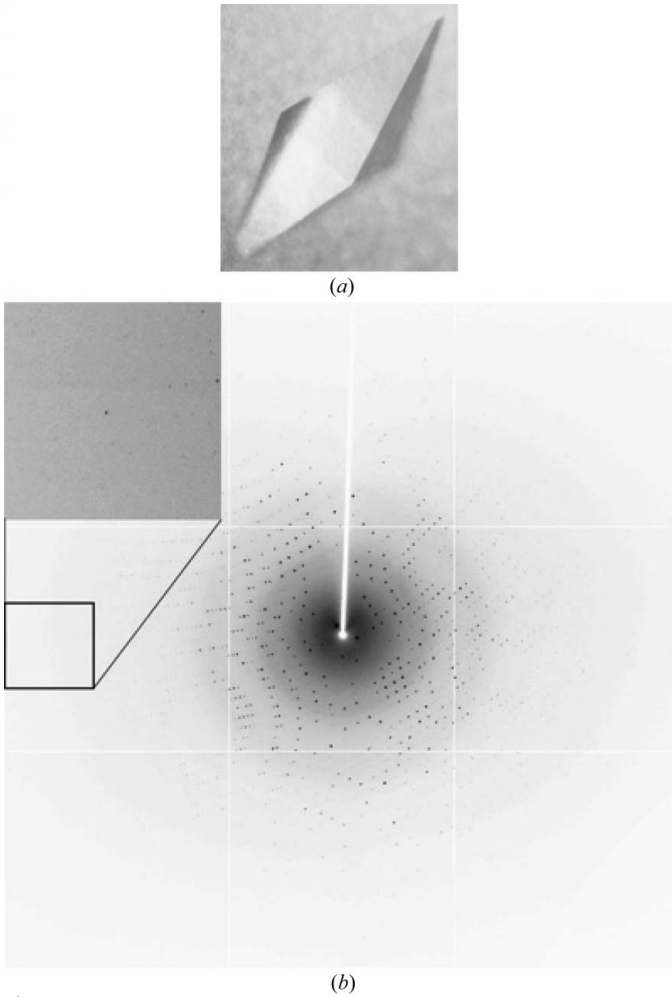

Figure 1
(*a*) Crystal of wild-type DDR in ADP-bound form. (*b*) An X-ray diffraction pattern from the DDR crystal in the ADP-bound form recorded at 100 K.

oscillation were recorded for each data set using the ADSC CCD detector system and were processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Fig. 1(*a*) shows a crystal of the ADP-bound form of DDR. The crystal parameters and data-processing statistics are summarized in Table 1. The crystals of the ADP-bound form of DDR belong to one of the orthorhombic space groups and the reflection conditions suggested space group $P2_12_12_1$. They diffract to 2.0 Å resolution with an $R_{\rm merge}$ of 8.7% and an overall completeness of 99.9%. A typical diffraction pattern from the crystal is shown in Fig. 1(*b*). Biochemical data suggest that the DDR molecule is composed of two α-subunits and two β-subunits. The Matthews volume $V_{\rm M}$ (Matthews, 1968) is 3.17 Å^3 Da^{-1}, corresponding to a solvent content of 61.2%, if $\alpha_2\beta_2$ (two $\alpha\beta$ heterodimers) is assigned to the asymmetric unit of the crystal. In the case of the nucleotide-free form, DDR crystals belong to the orthorhombic system, with space group $P2_12_12_1$. The Matthews coefficient and the solvent content are 3.33 Å^3 Da^{-1} and 63.1%, respectively, assuming the presence of one $\alpha_2\beta_2$ heterotetramer in the asymmetric unit. Both crystals were suitable for detailed structural analysis. In order to solve the structures of DDR in the ADP-bound and nucleotide-free forms by the multiple-wavelength anomalous dispersion (MAD) method, the expression, purification and crystallization of selenomethionine-substituted DDR are under way.

This work was supported in part by Grants-in-Aid for Scientific Research [(B) 13480195 and Priority Areas 753 to TT] from the Japan Society for Promotion of Science and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Bachovchin, W. W., Eagar, R. G. Jr, Moore, K. W. & Richards, J. H. (1977). *Biochemistry*, **16**, 1082–1092.
Forage, R. G. & Foster, M. A. (1979). *Biochim. Biophys. Acta*, **569**, 249–258.
Honda, S., Toraya, T. & Fukui, S. (1980). *J. Bacteriol.* **143**, 1458–1465.
Kajiura, H., Mori, K., Tobimatsu, T. & Toraya, T. (2001). *J. Biol. Chem.* **276**, 36514–36519.
Lee, H. A. Jr & Abeles, R. H. (1963). *J. Biol. Chem.* **238**, 2367–2373.
Liao, D.-I., Reiss, L., Turner, I. Jr & Dotson, G. (2003). *Structure*, **11**, 109–119.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mori, K., Bando, R., Hieda, N. & Toraya, T. (2004). *J. Bacteriol.* **186**, 6845–6854.
Mori, K., Tobimatsu, T., Hara, T. & Toraya, T. (1997). *J. Biol. Chem.* **272**, 32034–32041.
Mori, K. & Toraya, T. (1999). *Biochemistry*, **38**, 13170–13178.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Seifert, C., Bowien, S., Gottschalk, G. & Daniel, R. (2001). *Eur. J. Biochem.* **268**, 2369–2378.
Tobimatsu, T., Kajiura, H., Yunoki, M., Azuma, M. & Toraya, T. (1999). *J. Bacteriol.* **181**, 4110–4113.
Toraya, T. (2003). *Chem. Rev.* **103**, 2095–2127.
Toraya, T. & Fukui, S. (1977). *Eur. J. Biochem.* **76**, 285–289.
Toraya, T. & Fukui, S. (1982). *B_{12}*, edited by D. Dolphin, pp. 233–262. New York: John Wiley & Sons.
Toraya, T., Honda, S. & Fukui, S. (1979). *J. Bacteriol.* **139**, 39–47.
Toraya, T., Kuno, S. & Fukui, S. (1980). *J. Bacteriol.* **141**, 1439–1442.
Toraya, T. & Mori, K. (1999). *J. Biol. Chem.* **274**, 3372–3377.
Toraya, T., Shirakashi, T., Kosuga, T. & Fukui, S. (1976). *Biochem. Biophys. Res. Commun.* **69**, 475–480.
Ushio, K., Honda, S., Toraya, T. & Fukui, S. (1982). *J. Nutr. Sci. Vitaminol.* **28**, 225–236.