

Crystallization and preliminary X-ray analysis of the thymidylate kinase from *Mycobacterium tuberculosis*I. Li de la Sierra,^{a*} H. Munier-Lehmann,^b A. M. Gilles,^b O. Bárzu^b and M. Delarue^a^aUnité de Biochimie Structurale, Institut Pasteur, 28 Rue du Dr Roux, 75724, Paris CEDEX 15, France, and ^bLaboratoire de Chimie Structurale des Macromolécules, Institut Pasteur, 28 Rue du Dr Roux, 75724, Paris CEDEX 15, France

Correspondence e-mail: ines@pasteur.fr

Mycobacterium tuberculosis thymidylate kinase complexed with the substrate deoxythymidine monophosphate was crystallized in the hexagonal space group $P6_522$ or $P6_122$, with unit-cell parameters $a = b = 76.62$, $c = 134.38$ Å and one single monomer of 23 kDa in the asymmetric unit. Cryo-cooled crystals diffract at 1.94 Å resolution using synchrotron radiation.

Received 22 October 1999
Accepted 9 December 1999

1. Introduction

Thymidylate kinase (E.C. 2.7.4.9; ATP:dTMP phosphotransferase; TMPK) belongs to the nucleoside monophosphate kinase (NMPK) family and catalyzes the phosphorylation of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) using ATP as the phosphoryl donor in both *de novo* and salvage pathways of dTTP synthesis. Being essential for DNA synthesis and cellular growth (Anderson, 1973; Neuhard & Nygaard, 1996), this enzyme appeared to be an attractive target for the development of drugs against cancer (Jong & Campbell, 1984) and various viruses (Griffith, 1995; Darby, 1995). Variability in the structure and catalytic properties of TMPKs from various sources opened the possibility of designing specific inhibitors. Such inhibitors of *M. tuberculosis* thymidylate kinase (TMPK_{mt}) might be effective agents in limiting the spread of tuberculosis.

The three-dimensional structures of the NMPK family display a similar general topology. Nevertheless, several conformational changes can occur depending upon the occupancy of each of the nucleotide-binding sites (Vonnrhein *et al.*, 1995; Lavie *et al.*, 1997; Briozzo *et al.*, 1998). A high variability in the local structure and catalytic properties of this family of proteins was also observed (Brown *et al.*, 1995; Lavie *et al.*, 1997; Lavie, Konrad *et al.*, 1998; Lavie, Ostermann *et al.*, 1998; Chenal-Francisque *et al.*, 1999). The three-dimensional structures of TMPKs from yeast and *Escherichia coli* have been solved recently at high resolution (Lavie *et al.*, 1997; Lavie, Konrad *et al.*, 1998; Lavie, Ostermann *et al.*, 1998) and their analysis led the authors to propose a classification of TMPKs into two types according to the sequence variability of the catalytic region containing the phosphate binding loop (P-loop) and the region which covers part of the phosphate donor binding site (LID). The TMPK_{mt} sequence, however,

departs from both types in these two regions when compared with both yeast and *E. coli* enzymes. Moreover, the TMPK_{mt} kinetic properties show significant differences to the other two enzymes (Munier-Lehmann *et al.*, 2000). In order to explain these differences, we have crystallized the recombinant TMPK_{mt} in complex with dTMP and report here its preliminary X-ray study.

2. Materials and methods

The *tmk* gene of *M. tuberculosis* was cloned in *E. coli* and the overexpressed protein (a dimer of 214 amino acids per monomer) was purified by chromatography on blue-Sepharose and gel filtration (Munier-Lehmann *et al.*, 2000). The purity and the identity of the protein was controlled by SDS-PAGE and electrospray ionization mass spectrometry (ESI-MS). TMPK_{mt} was concentrated to 7–8 mg ml⁻¹ in 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5 mM DTT using a centrifugal filter device (Nanosep 10 K, Pallfiltron) at 277 K. This stock solution was conserved at 253 K for further crystallization experiments.

Crystallization trials were performed at 293 K by hanging-drop vapour diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 3–5 µl of TMPK_{mt}-dTMP protein solution (270–392 µM TMPK_{mt}, 2 mM dTMP, 1 mM DTT, 2 mM EDTA, 15 mM Tris-HCl pH 7.4) with an equal volume of the reservoir solution. The drops were equilibrated against 1 ml reservoir solution.

All data were collected from cryo-cooled crystals. Single crystals of TMPK_{mt}-dTMP were transferred using a Hampton Research loop into a stabilization solution which contained 25% glycerol. The loops were placed in a stream of nitrogen at 110 K to cryo-cool the crystal immediately prior to collecting data using an Oxford Cryosystems Cryostream or

Table 1
Data-collection conditions and statistics for the different diffraction data sets.

	Nat	Nat1	EtHgPO ₄
X-ray source (wavelength in Å)	In-house (1.5418)	ESRF ID14-3 (0.94)	LURE (1.28)
Detector	MAR imaging plate	MARCCD	MAR imaging plate
Crystal-to-detector distance (mm)	250	120 (250)†	200
Total rotation scan‡ (°)	71	80 (80)†§	82
Exposure time (s)	1800	5 (1)†	420
Space group	<i>P</i> ₆ ,22 or <i>P</i> ₆ ,22	<i>P</i> ₆ ,22 or <i>P</i> ₆ ,22	<i>P</i> ₆ ,22 or <i>P</i> ₆ ,22
Unit-cell parameters			
<i>a</i> , <i>b</i> (Å)	74.92	76.62	76.84
<i>c</i> (Å)	131.28	134.38	134.98
Resolution (Å)	20–3.6	40–1.9	12–3.0
Total number of reflections	21067	184620	22643
Number of unique reflections	2799	18761	4571
Multiplicity	7.5 (7.9)	9.5 (7.1)	5.0 (4.8)
Completeness (%)	99.9 (99.9)	100 (99.9)	90.7 (91.2)
<i>R</i> _{merge} ¶	0.131 (0.202)	0.052 (0.374)	0.094 (0.120)
<i>I</i> / <i>σ</i> (<i>I</i>)	22.0 (14.0)	37.4 (6.95)	26.1 (19.5)
<i>R</i> _{iso} ††	—	—	0.173

† Values for the low-resolution pass are in parentheses. ‡ All collections were carried out with an oscillation angle of 1°. § With three passages per oscillation angle. ¶ $R_{\text{merge}} = \frac{\sum_h \sum_i |I_{hi} - \langle I_h \rangle|}{\sum_h \sum_i I_{hi}}$, where I_{hi} is the i th observation of the reflection h , while $\langle I_h \rangle$ is the mean intensity of reflection h . †† $R_{\text{iso}} = \frac{\sum |F_{PH} - F_P|}{\sum F_P}$, where F_{PH} and F_P are the derivative and the native structure-factor amplitudes, respectively.

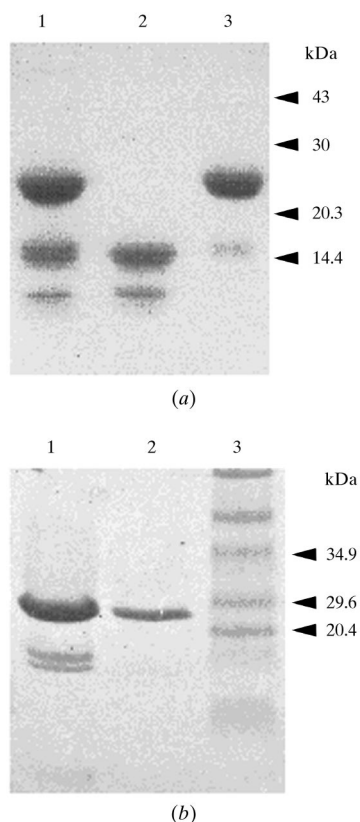


Figure 1
(a) SDS-PAGE (12.5%). Pharmacia molecular-weight markers are indicated on the right. Lane 1, TMPK_{mt} after three weeks at room temperature; lane 2, TMPK_{mt}-dTMP complex under the same conditions; lane 3, TMPK_{mt}-dTMP complex in presence of 1 mM EDTA and 0.5 mM DTT. (b) High Density Phast Gel (SDS, 8–25%). Lane 1, TMPK_{mt} protein solution used for crystallization; lane 2, washed crystals of the TMPK_{mt}-dTMP complex; lane 3, Biorad molecular-weight markers (molecular weights are indicated on the right).

were cryo-cooled and stored in liquid nitrogen for further data collection.

Several heavy-atom derivatives were screened in order to solve the structure by MIR methods. TMPK_{mt}-dTMP crystals were soaked in the stabilization solution with increased concentration of ammonium sulfate and heavy-atom reagents.

Diffraction data from the first native and the mercury-derivative crystals were processed using the *MARXDS* package (Kabsch, 1988a,b). Data from the second native crystal were processed with the *DENZO/SCALEPACK* suite of programs (Otwinowski & Minor, 1997). The *CCP4* package (Collaborative Computational Project, Number 4, 1994) was used to calculate observed structure factors and their intensity distribution (*TRUNCATE*) and scale native data to derivative data (*FHSCAL*). The Patterson maps were interpreted with the automated procedure developed in the program *HEAVY* (Terwilliger & Eisenberg, 1987) and the heavy-atom position was refined with *MLPHARE*

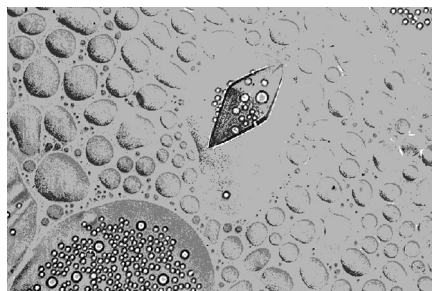


Figure 2
A typical crystal of the TMPK_{mt}-dTMP complex, measuring 0.3 mm in the longest dimension.

(*CCP4*); *DM* (*CCP4*) was used for solvent-flattening calculations.

3. Results and discussion

The TMPK_{mt} was of high purity. The mass of the protein in ESI-MS of $22\,635.89 \pm 2.23$ Da corresponds to that calculated from the sequence (22 634.58 Da) within the estimated error. Crystals of the TMPK_{mt}-dTMP complex grown using the same reservoir solution without EDTA and reducing agent were not stable and diffracted poorly. Biochemical analysis showed that in the presence of 2 mM dTMP, TMPK_{mt} was proteolyzed into two fragments (Fig. 1a), one corresponding to residues 1–148 (molecular mass of $15\,507.98 \pm 1.17$ Da determined by ESI-MS). When 1 mM EDTA and 0.5 mM DTT were included in buffers used for purification of the TMPK_{mt} (Fig. 1a) and crystallization of the TMPK_{mt}-dTMP complex, this phenomenon was suppressed (Fig. 1b).

The best crystals were obtained with a reservoir solution consisting of 35% ammonium sulfate, 2% PEG 2000, 0.1 M MES pH 6.0, 2 mM β-mercaptoethanol, 25 mM magnesium acetate. Suitable TMPK_{mt}-dTMP bipyrarnidal crystals appeared after about one month (Fig. 2) and have typical dimensions of up to $150 \times 150 \times 300$ μm. They belong to space group *P*₆,22 or *P*₆,22, with unit-cell parameters $a = b = 76.62$, $c = 134.38$ Å for the best single-crystal data set collected thus far. Assuming one TMPK_{mt}-dTMP monomer per asymmetric unit, the volume of the asymmetric unit divided by the protein molecular weight (V_m) value is 2.48 Å³ Da⁻¹ and the solvent content is 50%, which is near the average values found for most protein molecules (Matthews, 1968).

A cryo-cooled TMPK_{mt}-dTMP crystal diffracted X-rays to 3.6 or 1.9 Å using in-house and synchrotron X-ray radiation, respectively. Data were collected from two TMPK_{mt}-dTMP native crystals (Table 1). A complete data set for a first crystal diffracting to 3.6 Å was obtained using Cu Kα radiation from a rotating-anode generator. Two data sets for the second crystal were collected at high (1.9 Å) and low (3.68 Å) resolution using the ESRF synchrotron-radiation facility, Grenoble.

Diffraction data from an EtHgPO₄ heavy-atom derivative was obtained (Table 1) at 3 Å resolution using synchrotron X-ray radiation at the LURE laboratory, Orsay. The isomorphous difference Patterson map revealed one heavy-atom site. The heavy-atom position was refined at 9–3.8 Å reso-

lution with a phasing power of 1.1 and an R_{Cullis} value of 0.86 for acentric reflections. The values of the mean figure of merit (FOM) was 0.23 before and 0.59 after phase improvement through solvent flattening. A search for additional isomorphous heavy-atom derivatives is under way.

We thank A. Namane for ESI-MS and N. Expert-Bezancon for SDS-PAGE. We thank W. Shepard and X. Perez at LURE and S. Arzt and W. Burmeister at ESRF for help with synchrotron data collection. This work was supported by a post-doctoral fellowship from the EEC and grants from the EEC, the Institut Pasteur and the Centre National de la Recherche Scientifique (URA 1129).

References

- Anderson, E. P. (1973). *The Enzymes*, edited by P. D. Boyer, Vol. 8, pp. 49–96. New York: Academic Press.
- Briozzo, P., Golinelli-Pimpaneau, B., Gilles, A.-M., Gaucher, J.-F., Burlacu-Miron, S., Sakamoto, H., Janin, J. & Barzu, O. (1998). *Structure*, **6**, 1517–1527.
- Brown, D. G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P. J., Melitz, C., Summers, W. C. & Sanderson, M. R. (1995). *Nature Struct. Biol.* **2**, 876–881.
- Chenal-Francisque, V., Tourneux, L., Carniel, E., Christova, P., Li de la Sierra, I. M., Bâzru, O. & Gilles, A.-M. (1999). *Eur. J. Biochem.* **265**, 112–119.
- Collaborative Computational Project, Number 4. (1994). *Acta Cryst. D***50**, 760–763.
- Darby, G. K. (1995). *Antivir. Chem. Chemother.* **6**, 54–63.
- Griffith, P. D. (1995). *Antivir. Chem. Chemother.* **6**, 191–209.
- Jong, A. Y. & Campbell, J. L. (1984). *J. Biol. Chem.* **259**, 14394–8.
- Kabsch, W. (1988a). *J. Appl. Cryst.* **21**, 67–71.
- Kabsch, W. (1988b). *J. Appl. Cryst.* **21**, 916–924.
- Lavie, A., Konrad, M., Brundiers, R., Goody, R. S., Schlichting, I. & Reinstein, J. (1998). *Biochemistry*, **37**, 3677–3686.
- Lavie, A., Ostermann, N., Brundiers, R., Goody, R. S., Reinstein, J., Konrad, M. & Schlichting, I. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 14045–14050.
- Lavie, A., Vetter, I. R., Konrad, M., Goody, R. S., Reinstein, J. & Schlichting, I. (1997). *Nature Struct. Biol.* **4**(8), 601–604.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Munier-Lehmann, H., Chaffotte, A. & Labesse, G. (2000). In preparation.
- Neuhard, J. & Nygaard, P. (1996). *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, edited by F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umberger, Vol. 1, pp. 580–599. Washington DC: American Society for Microbiology.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Terwilliger, T. C. & Eisenberg, D. (1987). *Acta Cryst.* **A43**, 6–13.
- Vonrhein, C., Schlauderer, G. J. & Schulz, G. E. (1995). *Structure*, **3**, 483–490.