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The 2.2 Å resolution structure of thermolysin (TLN) crystallized in the presence of potassium thiocyanate

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A new crystallization protocol for thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus* is presented. After dissolving the protein in the presence of KSCN, which avoids the use of DMSO and CsCl, crystals were obtained following the salting-in method. Crystal cell parameters are isomorphous with those previously reported from DMSO/CsCl mixtures. The new SCN^- crystal structure has been analyzed. It shows the presence of one thiocyanate ion in the catalytic site and several rearrangements in the S_1 and S_2 subsites. These results are in agreement with the measurements of Inouye *et al.* [(1998), *J. Biochem. (Tokyo)*, **123**, 847–852], who observed in solution that the solubility of TLN, which is particularly poor in low ionic strength solutions, increases dramatically in the presence of several neutral salts. The results reported here suggest possible explanations for the solubility increase and for the inhibitory effects of high SCN^- concentrations on thermolysin activity.

1. Introduction

Thermolysin (TLN; EC 3.4.24.27) is a thermostable metalloendopeptidase of molecular weight 36 400 Da which is isolated from *B. thermoproteolyticus*. Crystallographic structures of TLN complexed with a variety of inhibitors have provided evidence for the binding mode of substrates and the catalytic mechanism (Matthews, 1988). However, the role of DMSO and ionic strength in the crystallization process still remains unclear.

Usually, thermolysin and/or its complexes are crystallized after dissolving TLN at high concentration (>200 mg ml^{-1}) in a mixture of DMSO and calcium acetate or CsCl by vapour-diffusion of hanging drops against a low-concentration reservoir (Matthews *et al.*, 1972). In this particular case, the water vapour diffuses from the reservoir to the drop, whose volume increases. The supersaturation of TLN, a prerequisite for crystallization, is reached because of the low solubility of TLN in the absence of DMSO or salts.

The TLN structure, which usually retains the Val-Lys dipeptide, has been refined at 1.6 Å resolution and shows the presence of a DMSO molecule (Holmes & Matthews, 1982).

Following this standardized method, other structures of TLN cocrystallized with thiolate inhibitors were obtained. They also form a complex with a DMSO molecule (Gaucher *et al.*, 1999).

The mechanism of crystallization implies a salting-in effect. Indeed, at low ionic strength, the concentration of soluble TLN in the presence of precipitate is measured as 0.16 mg ml^{-1} at pH 7.0, 278 K, 0.2 *M* sodium acetate (Sazaki *et al.*, 1993). It dramatically increases in the presence of 45%(*v*/*v*) DMSO and 1.4 *M* calcium acetate or 2.5 *M* CsCl at pH 7.2 (Matthews *et al.*, 1972; Holmes & Matthews, 1982). More recently, Inouye *et al.* (1998) observed the same increase of solubility in the presence of several neutral salts without DMSO.

The effect of salts is complex: Inouye *et al.* (1998) reported that salt concentration affected both the solubility and the activation of TLN without any apparent correlation between the two effects.

Firstly, the effect of the anion and cation on solubility (here defined as the soluble protein concentration in the presence of precipitate) was analyzed. The solubility is affected more by anions than cations. Anions dramatically increase the solubility following the reverse order of the Hofmeister series, with an effectiveness $SCN^- > I^- > ClO_4^- > NO_3^- > Br^- > Cl^- > CH_3COO^-$. The effectiveness of cations follows the order $Na^+ > K^+ > Li^+$. The authors suggested that this last effect, which did not follow the Hofmeister series, is induced by specific interactions between ions and the enzyme.

Secondly, the effect of neutral salts on the reaction rate of TLN-catalyzed hydrolysis of FAGLA [3-(2-furyl)acryloyl-L-Leu-L-Ala] was investigated. The k_{cat}/K_M increased in an apparent exponential fashion in the presence of NaCl, NaBr, KCl and NaBr. In the presence of NaSCN, the activity reaches its optimum at 1 *M* and decreases at higher concentrations.

In the present paper, we have solubilized TLN to high concentration in KSCN solution and crystallized it by vapour diffusion against a low-concentration reservoir. We are focusing on the specific interactions between thiocyanate and TLN. The crystal structure was then refined to disclose the corresponding interactions.

2. Crystallization, data collection and processing

Thermolysin was purchased from Sigma–Aldrich Co. Since the commercial enzyme powder contains salts, it was first purified by reprecipitation: 3 mg of lyophilized TLN was suspended in 100 µl of water and centrifuged at 7200*g*. The pellet was freeze-dried and redissolved in 15 µl of solution *A* [50 m*M* tris(hydroxymethyl)aminomethane–HCl buffer pH 8.3, 1 *M* KSCN, 20 m*M* $CaCl_2$]. The enzyme concentration was estimated using an ε_{280} of 56 000 M^{-1} cm^{-1}. 0.5 µl drops (87 mg ml^{-1} TLN) were equilibrated against 1 ml reservoirs of diluted solution *A* in water. The best conditions were reached with a mixture of 740 µl solution *A* and 260 µl water. Hexagonal crystals appeared in one week at 277 K. The reservoirs were then diluted with 0.3 ml water. Crystals grew to their final size within two more weeks.

Diffraction data were recorded at the LURE synchrotron facility, Orsay, France at 283 K on the DW21b wiggler beamline (1.375 Å wavelength; 300 mm diameter MAR Research image-plate detector system). Data were processed with *MOSFLM* (Leslie, 2001) interfaced with the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

The crystal space group is $P6_122$, with unit-cell parameters $a = b = 93.17$, $c = 130.63$ Å, one molecule per asymmetric unit and 39% solvent. 58 898 reflections were measured and reduced to 15 898 unique reflections [R_{merge} = 8.6%, $I/\sigma(I)$ = 7.3], with 92.2% completeness within the range 18.5–2.18 Å [R_{merge} = 24.1% and 82.7% completeness in the last resolution shell 2.30–2.18 Å, with $I/\sigma(I)$ = 2.3].

3. Refinement

The starting model was the native TLN structure crystallized in a mixture of DMSO and CsCl (PDB code 8tln; Holmes & Matthews, 1982). The thermal parameters of atoms were set to the average value deduced from the Wilson plot ($\langle B \rangle$ = 18.6 Å^2).

Because the crystal was isomorphous with 8tln, the refinement began with a rigid-body least-squares refinement (*CNS* 1.1; Brünger *et al.*, 1998), followed by refinements of the atomic positions by energy minimization at the resolution of 2.25 Å.

At this stage, grouped thermal parameters (two for each residue) were refined and 134 water molecules were progressively added in several cycles of energy minimization and grouped thermal parameter refinements.

In the structure, a Fourier difference map reveals a shift of Tyr157 in the catalytic active site and the presence of bottle-shaped electron density near the catalytic zinc.

The resolution was then extended to 2.18 Å and individual thermal parameters were refined. The bottle-shaped density, which was initially interpreted as two discrete water molecules, showed unrealistically low *B* factors. It was then modelled as an SCN^- ion, which now perfectly fits the electron density.

Refinement ended with an overall *R* factor of 16.3% and R_{free} = 21.4%. The statistics of the refinement are given in Table 1.

Table 1
Statistics of the TLN/SCN^- refinement.

Values in parentheses are for the highest resolution bin (2.32–2.18 Å).

Resolution range (Å)	19–2.18
Completeness (%)	92.4 (83.9)
R factor/R_{free} (%)	16.3/21.4 (18.3/24.2)
Estimated coordinate error (Luzzati plot/σ) (Å)	0.18/0.15
RMS deviations from ideal values	
Bond lengths (Å)	0.007
Bond angles (°)	1.3
Dihedral angles (°)	22.8
Improper angles (°)	0.73
Isotropic thermal factor restraint (RMS/σ)	
Main-chain bond (Å^2)	1.44/1.50
Main-chain angle (Å^2)	2.02/2.00
Side-chain bond (Å^2)	2.57/2.00
Side-chain angle (Å^2)	3.51/2.50

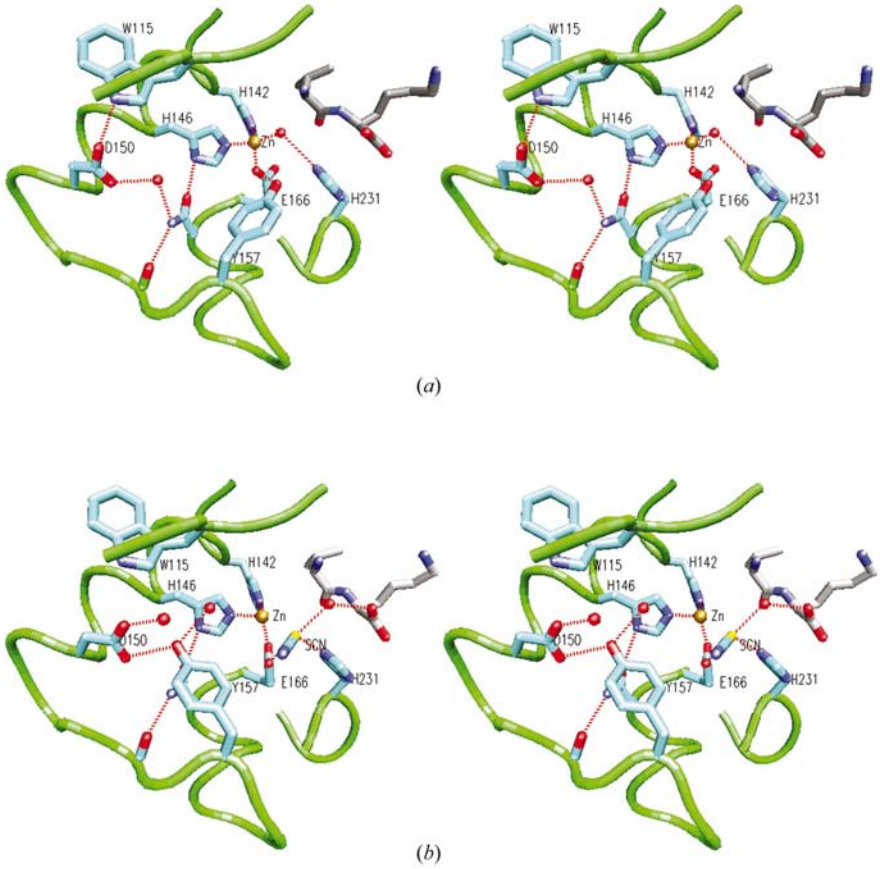

Figure 1
Stereoimages illustrating the mode of binding of the SCN ion to TLN and the rearrangement of the subsite S1–S2. (*a*) Partial structure of the active site of TLN crystallized in the presence of DMSO/CsCl. (*b*) Partial structure of the same active site of TLN crystallized in the presence of KSCN. Tyr157 rotates about the χ_1 angle toward Asp150. The SCN ion tightly binds His231, Glu166 and a water molecule (*VMD*; Humphrey *et al.*, 1996).

4. Results and discussion

The use of thiocyanate as a crystallizing agent has been popularized in the last decade following the work of Riès-Kautt & Ducruix (1989, 1991). The efficiency of thiocyanate in crystallizing basic pI proteins has been demonstrated in the cases of erabutoxin B (Saludjian *et al.*, 1992) and lysozyme (Vaney *et al.*, 2001). In most cases, a thiocyanate ion is retained within the crystal structure and shows strong interactions with positively charged residues (Hamiaux *et al.*, 1999).

In the refined structure of TLN/SCN^-, 316 residues, 180 water molecules, four Ca atoms and one Zn atom were characterized. It also includes, in addition to the usual Val-Lys dipeptide located in the S1′–S2′ catalytic subsites, one well defined thiocyanate.

The TLN structure itself is virtually identical to the structure obtained from DMSO/CsCl. The main differences are observed in the active site: the hydrogen-bonding network between residues implied in the catalysis mechanism is disturbed by the SCN^- ion located in the vicinity of the zinc (Fig. 1).

In TLN crystallized from DMSO/CsCl, the zinc ligands (His146, His142, Glu166 and a water molecule) form a tetragonal coordination. In the new structure, the zinc coordination is no longer symmetrical: the water molecule is shifted away by the SCN^-. The S atom moves to a distance of 3.1 Å from the Zn^{2+}. Consequently, the Glu166 carboxylate is displaced and its OE1 atom now binds the zinc instead of OE2.

Surprisingly, the thiocyanate S atom is strongly coordinated to TLN residues: it is 2.6 Å from Glu166 OE2, which seems protonated. It is also 2.4 Å from the NE2 atom of the positively charged His231 and 3.0 Å from a water molecule.

In DMSO/CsCl structures, the hydroxyl group of Tyr157 is tightly hydrogen bonded to Glu166 (2.6 Å). In the presence of SCN, this conformation is no longer possible because of a steric clash with the thiocyanate. Tyr157 rotates about χ_1 and is now hydrogen bonded to Asp150 (2.9 Å) and a water molecule (3.0 Å).

The Asp150 residue is also shifted: its short hydrogen bond with Trp115 (2.8 Å), as observed in the DMSO/CsCl structure, is now disrupted and its carboxylate group binds a water molecule (2.7 Å) and the hydroxyl group of Tyr157.

The new structure must be related to some properties observed in solution of TLN in the presence of SCN^- and neutral salts. Firstly, the solubility was shown to increase in the presence of SCN^-. The calculated isoelectric point of TLN, taking into account the four Ca^{2+} and the Zn^{2+} coions, is 6.8. The charge on TLN is then −2 and −5 at pH 7.2 and 8.3, respectively. Consequently, the specific interaction of SCN^- and TLN contributes to significantly increase the net negative charge of the protein. This leads to increased solubility.

Secondly, the enzymatic activity is enhanced by the ionic strength (Inouye, 1992; Inouye *et al.*, 1998), but in the case of thiocyanate and to a lesser extent with LiBr and LiCl, the salt has an inhibitory effect at high concentration. In the new structure described here, the Tyr157 conformation and the presence of SCN^- in the catalytic site prevent the substrate binding and consequently might be the reason for the competitive inhibitory effect observed by Inouye and coworkers.

References

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.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Gaucher, J. F., Selkti, M., Tiraboschi, G., Prangé, T., Roques, B. P., Tomas, A. & Fournie-Zaluski, M. C. (1999). *Biochemistry*, **38**, 12569–12576.

Hamiaux, C., Prangé, T., Riès-Kautt, M., Ducruix, A., Lafont, S. & Veesler, S. (1999). *Acta Cryst.* D**55**, 103–113.

Holmes, M. A. & Matthews, B. W. (1982). *J. Mol. Biol.* **160**, 623–639.

Humphrey, W., Dalke, A. & Schulten, K. (1996). *J. Mol. Graph.* **14**, 33–38, 27–28.

Inouye, K. (1992). *J. Biochem. (Tokyo)*, **112**, 335–340.

Inouye, K., Kuzuya, K. & Tonomura, B. (1998). *J. Biochem. (Tokyo)*, **123**, 847–852.

Leslie, A. G. W. (2001). *MOSFLM Program For Autoindexing and Integrating X-ray Diffraction Data, Version* 6.11. MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England.

Matthews, B. W. (1988). *Acc. Chem. Res.* **21**, 333–340.

Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P. & Dupourque, D. (1972). *Nature (London)*, **238**, 37–43.

Riès-Kautt, M. & Ducruix, A. (1989). *J. Biol. Chem.* **264**, 745–748.

Riès-Kautt, M. & Ducruix, A. (1991). *J. Cryst. Growth*, **109**, 20–25.

Saludjian, P., Prangé, T., Navaza, J., Ménez, R., Guilloteau, J. P., Riès-Kautt, M. & Ducruix, A. (1992). *Acta Cryst.* B**48**, 520–531.

Sazaki, G., Ooshima, H., Kato, J., Harano, Y. & Hirokawa, N. (1993). *J. Cryst. Growth*, **130**, 357–367.

Vaney, M. C., Broutin, I., Retailleau, P., Douanga-math, A., Lafont, S., Hamiaux, C., Prangé, T., Ducruix, A. & Riès-Kautt, M. (2001). *Acta Cryst.* D**57**, 929–940.