Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization, preliminary X-ray analysis and biophysical characterization of HPr kinase/phosphatase of *Mycoplasma pneumoniae*

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Received 5 October 2001
Accepted 13 December 2001

The *Mycoplasma pneumoniae* HPr kinase/phosphatase (HPrK/P) is a member of a large family of enzymes which are central to carbon regulation in Gram-positive bacteria. The full-length *M. pneumonia* HPrK/P was crystallized from solutions of polyethylene glycol 8000 and KCl or NaCl which also contained the non-hydrolysable ATP analog adenosine 5′-[β,γ-methylene]triphosphate (AMPPCP). The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 117.1, b = 127.7, c = 170.7 Å. A complete X-ray intensity data set has been collected and processed to 2.50 Å resolution. The slow self-rotation function revealed the presence of a sixfold axis. Dynamic light-scattering (DLS) experiments indicated a molecular weight of 197 kDa for HPrK/P in the absence of AMPPCP and of 217 kDa in the presence of the ATP analog. Thus, the biophysical and crystallographic data suggest that HPrK/P is a functional hexamer that undergoes an ATP-binding-induced conformational change.

1. Introduction

Mycoplasma species belong to the low-guanine/cytosine (GC) family of Gram-positive bacteria and, with a genome size which ranges from 0.58 to 1.35 Mbp, are the smallest self-replicating organisms (Razin *et al.*, 1998). The fastidious growth requirements of *Mycoplasma* species result from a streamlined genome and dictate their parasitic lifestyle. Known hosts include plants, fish, birds, insects and mammals (Razin *et al.*, 1998). *Mycoplasma* species in humans and animals are usually found attached to the surfaces of epithelial cells; nevertheless, they also seem to be capable of entering the intracellular space. The latter may enable them to escape the immune response as well as selective antibiotic treatment (Rosengarten *et al.*, 2000). Their impact on human health and the somewhat mysterious molecular basis of their pathogenicity makes them of particular interest, especially in light of the available genomic information (Rosengarten *et al.*, 2000).

The genomes of *M. genitalium* and the closely related *M. pneumoniae* have been sequenced (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). The genome-sequencing projects of the two *Mycoplasma* species revealed that these organisms possess few genes encoding regulatory proteins. For example, they possess only a single sigma factor and lack two-component regulatory systems (Dybvig & Voelker, 1996; Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Razin *et al.*, 1998). Also, the genomic sequences of *M. pneumoniae* and *M. genitalium* revealed the existence of orthologs of HPrK/P (HPr kinase/phosphatase) in these organisms. HPrK/P is the key enzyme in the regulation of carbon catabolism in other more complex low-GC Gram-positive bacteria such as *Bacillus subtilis*, *Enterococcus faecalis* or *Lactobacillus casei* (Dossonnet *et al.*, 2000; Galinier *et al.*, 1998; Kravanja *et al.*, 1999; Reizer *et al.*, 1998). In these organisms, HPrK/P responds to the metabolic state of the cell by phosphorylating or dephosphorylating its substrate, the heat-stable protein (HPr) of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS). The phosphorylation state of HPr in turn signals the metabolic state of the cell; the different forms of HPr are recognized by proteins in several distinct regulatory systems (Stülke & Hillen, 1998). Presumably, the HPrK/P orthologs found in *Mycoplasma* species also play an important role in the regulation of energy metabolism in these organisms.

In vitro biochemical analysis of *M. pneumoniae* HPrK/P revealed that the enzyme exhibits kinase activity at low ATP concentrations ([ATP] > 1 μM) and requires inorganic phosphate ([P_i] > 1 mM) for phosphatase activity (Steinhauer *et al.*, 2002). In contrast, HPrK/P from *B. subtilis* requires high ATP concentrations ([ATP] > 100 μM) for kinase activity but low inorganic phosphate ([P_i] > 200 μM) to exhibit phosphatase activity (Hanson *et al.*, 2002; Jault *et al.*, 2000; Monedero *et al.*, 2001). Therefore, the intrinsic activities of the HPrK/P from *M. pneumoniae*

and *B. subtilis* differ: HPrK/P from *M. pneumoniae* is primarily a kinase, while the *B. subtilis* enzyme is by default a phosphatase. Besides having different intrinsic activities, both enzymes are regulated by ATP, inorganic phosphate and the glycolytic intermediate fructose-1,6-bisphosphate (Hanson *et al.*, 2002; Jault *et al.*, 2000; Kravanja *et al.*, 1999; Reizer *et al.*, 1998; Steinhauer *et al.*, 2002). This regulation of HPrK/P by low-molecular-weight effectors is an indication of its role as a sensor of the metabolic state of the cell and points to a mechanism for the regulation of carbon catabolism.

In addition to their enzymatic differences, gel-filtration experiments suggest that *M. pneumoniae* HPrK/P is active as a hexamer while the enzyme from *B. subtilis* is active as an octamer (Steinhauer *et al.*, 2002). Recently, the crystal structure of an N-terminal truncation mutant of the *Lactobacillus casei* HPrK/P, which is missing residues 1–127, was determined and found to be hexameric (Fieulaine *et al.*, 2001). However, no three-dimensional structural data are yet available for any full-length HPrK/P enzyme. In order to obtain a more detailed understanding of the structural organization and mechanism of HPrK/P, we carried out further biophysical characterization as well as the crystallization of the *M. pneumoniae* enzyme. Here, we describe the crystallization of HPrK/P and experiments designed to address possible structural changes of the protein upon ATP binding.

2. Materials and methods

2.1. Purification of HPrK/P

HPrK/P from *M. pneumoniae* carrying an N-terminal hexahistidine sequence was purified in buffer containing 10 m*M* Tris–HCl pH 7.5, 10 m*M* β-mercaptoethanol and 0.6 *M* NaCl. The protein was bound to a nickel-chelate resin and eluted with a gradient of imidazole in purification buffer as described previously (Steinhauer *et al.*, 2002). In order to remove the imidazole, the eluate was dialyzed against purification buffer. The dialyzed protein was then concentrated to 7 mg ml^{-1} as determined by Bradford assay.

2.2. Preparation of selenomethionine-substituted HPrK/P

A selenomethionine-substituted HPrK/P was prepared by transforming the methionine auxotrophic strain *E. coli* B834 (Novagen) with plasmid pGP204 carrying the *M. pneumoniae hprK* gene (Steinhauer *et al.*, 2002). Cells were grown in supplemented M9 minimal medium containing selenomethionine at a final concentration of 80 mg l^{-1} (Doublié, 1997). Cells were grown at 310 K in the dark to prevent light-induced oxidation of selenomethionine. Expression of the recombinant protein was induced by the addition of 1 m*M* IPTG to logarithmically growing cultures (OD_{600} of 0.6). The selenomethionine-substituted protein was purified as described above except for the addition of 15 m*M* β-mercaptoethanol to the purification buffer throughout purification. The final yield of the selenomethionine-substituted HPrK/P was 12 mg l^{-1}.

2.3. DTNB titration of HPrK/P

Titration of the thiols of HPrK/P with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was carried out in a buffer containing 10 m*M* Tris pH 7.2 and 0.6 *M* NaCl at room temperature; DTNB was dissolved in 0.1 *M* phosphate buffer pH 7.2 (Ellman, 1959). The reaction was followed spectrophotometrically at 410 nm and was complete after approximately 100 min. The amount of labelled thiol groups per HPrK/P monomer was calculated using the extinction coefficient of TNB^{2-} at λ = 410 nm (Riddles *et al.*, 1983). Only freshly purified HPrK/P devoid of reducing agent was used for the DTNB titration. Following the reaction with DTNB, labeled HPrK/P was used for activity assays, which were carried out as described in Steinhauer *et al.* (2002). Briefly, 20 μ*M* (His_6)HPr or (His_6)HPr(Ser-P) were incubated with 350 n*M* HPrK/P in assay buffer (10 m*M* $MgCl_2$, 25 m*M* Tris–HCl pH 7.6, 1 m*M* dithiothreitol) in a final volume of 20 µl. After 15 min incubation at 310 K, the enzyme was inactivated. The proteins were analyzed using 10% native PAGE.

2.4. Dynamic light-scattering experiments

Dynamic light-scattering studies on HPrK/P were performed using a DynaPro-801 Dynamic Light Scattering Instrument (Protein Solutions Inc.). Protein samples at 1.5 or 1 mg ml^{-1} were prepared in solutions of 25 m*M* Tris–HCl pH 7.6 containing either 600, 360 or 200 m*M* NaCl and 6 m*M* β-mercaptoethanol in the presence or absence of 1 m*M* adenosine 5′-[β,γ-methylene] triphosphate (AMPPCP), a non-hydrolysable ATP analog, and 10 m*M* $MgCl_2$. Prior to the experiment, all protein samples were filtered through 0.1 µm Anotop 10 filters (Whatman) to eliminate any large aggregates. All data were analyzed using *AutoPro PC* (Protein Solutions Inc.) software. The reported values are averages of five scans of 2 min each.

2.5. Crystallization of HPrK/P

Crystals of HPrK/P were grown at room temperature in hanging drops by the vapor-diffusion method (McPherson, 1999). The crystallization solution contained 4% polyethyleneglycol 8000, 20 m*M* $MgCl_2$, 50 m*M* Tris–HCl pH 7.6 and 0.8 *M* KCl or NaCl. In a typical hanging-drop experiment, 3 µl HPrK/P (7 mg ml^{-1}) in 10 m*M* Tris–HCl pH 7.5, 0.6 *M* NaCl and 1 m*M* AMPPCP were mixed with 3 µl of the crystallization solution and equilibrated over a 0.8 ml reservoir at room temperature. Crystals took about a week to appear and grew for three additional weeks. Crystals of the selenomethionine-substituted protein were obtained under the same conditions as described for the native HPrK/P, except that the crystallization solution contained 0.9 *M* NaCl and the protein concentration used for crystallization was 5 mg ml^{-1}.

2.6. Cryoprotection and X-ray intensity data collection

Cryocooling conditions for crystals of both the native and selenomethionine-substituted HPrK/P proteins were obtained by modifying a method that significantly changes the ionic strength of the crystallization liquor while attempting to maintain its osmolality (McRee, 1999). The scheme involves passing the crystal through four drops (5 µl each) of increasing glycerol concentration and decreasing salt concentration. This stepwise approach is necessary because the crystals are destroyed if placed directly in the final cryoprotectant solution. Specifically, the first transfer from the crystallization solution (20 m*M* $MgCl_2$, 50 m*M* Tris–HCl pH 7.6, 4% PEG 8000 and 0.8 *M* NaCl; ∼2.34 osmol kg^{-1}) removes the PEG 8000, decreases the NaCl to 0.5 *M* and adds 10% glycerol (∼2.35 osmol kg^{-1}). The second transfer further decreases the salt concentration to 0.2 *M* and increases the glycerol to 20% (∼3.45 osmol kg^{-1}). Incubation in the third and fourth transfer drops simply increases the glycerol in 5% increments to a final concentration of 30% (∼4.55 and ∼5.65 osmol kg^{-1}, respectively). Transfer drop four pre-empts formation of ice. The crystal is then placed in the nitrogen cryostream. Depending upon the size of the crystal, it is incubated for 1–3 min per drop; larger crystals are soaked for 3 min. X-ray intensity data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beamline BL 9.2 using a Quantum 4 CCD

and $\lambda = 0.88560$ Å. All data were collected at 100 K. The intensity data were processed with *d*TREK* v5.5i (Pflugrath, 1999).

3. Results and discussion

3.1. DTNB titration of HPrK/P

In order to determine the solvent-accessible thiol content of the enzyme, which might provide possible heavy-atom derivative sites for mercury, titration of $(His)_6$HPrK/P with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was carried out and monitored spectrophotometrically (Ellman, 1959). Repeated titration of freshly purified $(His)_6$HPrK/P with DTNB revealed a thiol content of 1.8 per monomer, indicating that both cysteines of the *M. pneumoniae* enzyme are solvent-accessible thiols rather than disulfide bonded or buried.

To investigate whether the cysteines are crucial for enzyme activity, we carried out enzyme-activity assays following DTNB titration of HPrK/P. The kinase activity of HPrK/P was tested in the presence of 10 μ*M* ATP and in the presence or absence of 10 m*M* fructose-1,6-bisphosphate (FBP); FBP has been shown to stimulate the kinase activity of some HPrK/P proteins (Dossonnet *et al.*, 2000; Galinier *et al.*, 1998; Jault *et al.*, 2000; Kravanja *et al.*, 1999; Reizer *et al.*, 1998), but to exhibit no stimulatory effect on the kinase activity of *M. pneumoniae* HPrK/P (Steinhauer *et al.*, 2002). The results obtained for the DTNB-labeled HPrK/P also did not show any stimulatory effect of FBP on kinase activity (Fig. 1*a*). Furthermore, the phosphorylation efficiencies of the non-labeled HPrK/P protein and HPrK/P following DTNB titration were similar. However, in the presence of 50 μ*M* ATP and 5 m*M* P_i, addition of 1 m*M* FBP to the reaction mixture prevented kinase inhibition by P_i (Steinhauer *et al.*, 2002). There was also no difference in phosphatase activity or its regulation upon labeling of *M. pneumoniae* HPrK/P when compared with the unlabeled enzyme (Fig. 1*b*).

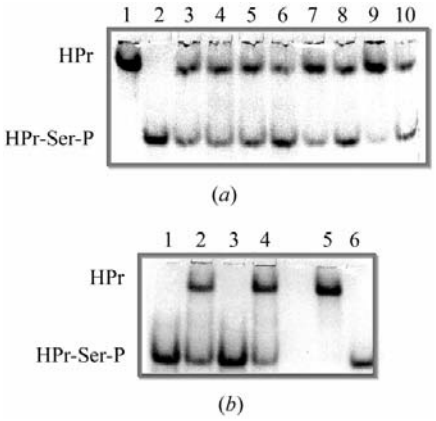

Figure 1
Enzyme activity of HPrK/P following DTNB labeling. (*a*) Kinase activity of HPrK/P. Lanes 1 and 2 contain as controls (His_6)HPr and (His_6)HPr(phosphoSer46), respectively. Lanes 3–6 show HPrK/P kinase activity in the presence of 10 μ*M* ATP and in the presence (even numbers) or absence (odd numbers) of 10 m*M* fructose-1,6-bisphosphate (FBP). In lanes 3 and 4 the result of the kinase reaction in the presence of DTNB-labeled HPrK/P is shown, whereas lanes 5 and 6 represent the reference reaction including unlabelled HPrK/P. Lanes 7–10 show HPrK/P kinase activity in the presence of 50 μ*M* ATP and 5 m*M* P_i and in the presence (even numbers) or absence (odd numbers) of FBP (1 m*M*). Lanes 7 and 8 show the reaction with the DTNB-labeled HPrK/P; lanes 9 and 10 are the reference experiments. (*b*) Phosphatase activity of HPrK/P. Lanes 1–4 show HPrK/P phosphatase activity in the presence (even numbers) or absence (odd numbers) of 5 m*M* P_i. In lanes 1 and 2 the reaction mixture included HPrK/P following DTNB titration. Lanes 3 and 4 show the respective reactions for the unlabeled HPrK/P. Lanes 5 and 6 are controls containing (His_6)HPr and (His_6)HPr(phosphoSer46), respectively.

3.2. Dynamic light-scattering experiments

Previous size-exclusion chromatography experiments indicated that *M. pneumoniae* HPrK/P is an hexamer in solution (Steinhauer *et al.*, 2002). Dynamic light-scattering (DLS) experiments were carried out to further characterize the oligomeric properties of the enzyme in the presence and absence of AMPPCP. DLS offers the advantage of studying a macromolecule in its true solution state; chromatography or gel matrices, which can confound interpretations of data by their non-specific interaction with protein, are not present. DLS also indicates the aggregation state of a protein under a given set of experimental conditions. This information was useful for designing crystallization experiments.

A 1 mg ml^{-1} (27 μ*M* on a monomer basis) solution of HPrK/P in the presence of 0.6 *M* NaCl was monodisperse and had an estimated molecular weight of 197 kDa. A reduction of the salt concentration to 360 or 200 m*M* and an increase in the protein concentration to 1.5 mg ml^{-1} (41 μ*M*) gave similar results. Addition of 1 m*M* AMPPCP resulted in an increase of the estimated apparent molecular weight of HPrK/P to 217 kDa. Therefore, the data obtained by dynamic light scattering indicate that the oligomeric state of apo $(His)_6$HPrK/P is either a pentamer (184 kDa) or a hexamer (221 kDa), whereas the ATP-bound enzyme is clearly a hexamer.

The change in the apparent molecular weight detected upon nucleotide binding has two possible explanations. The first is that in the absence of nucleotides HPrK/P exists as a pentamer, which upon binding ATP adds an additional monomer to the complex, resulting in the formation of a hexamer. The second and more plausible interpretation is that HPrK/P takes a more compact conformation in the absence of ATP, thereby leading to a decreased hydrodynamic radius, which is detected as a smaller molecular weight. Thus, it is very likely that the *M. pneumoniae* HPrK/P forms hexamers even in the absence of nucleotides and can undergo significant nucleotide-induced conformational changes.

Table 1
Data-collection statistics for HPrK/P from *M. pneumoniae*.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	30.0–2.49 (2.59–2.49)
No. of observations	336892 (34222)
No. of unique observations	86543 (9077)
Multiplicity	3.9 (3.8)
Completeness (%)	95.6 (91.0)
Mean $I/\sigma(I)$	16.8 (4.0)
R_{iso}†	0.068 (0.433)

† $R_{iso} = \sum |I_i - I_{ave}| / I_{avg}$.

3.3. X-ray data collection and analysis

Orthorhombic crystals of native HPrK/P and its selenomethionine derivative were obtained as described in §2 (Fig. 2). X-ray intensity data were collected at cryogenic temperatures to a resolution greater than 2.5 Å. The data were processed with *d*TREK* v5.5i (Table 1) (Pflugrath, 1999).

The diffraction pattern displayed 2/*m* 2/*m* 2/*m* Laue symmetry. Analysis of the *hk*0, *h*0*l* and 0*kl* zones revealed the systematic

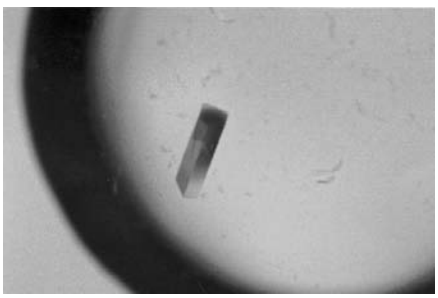

Figure 2
Crystal of *M. pneumoniae* HPrK/P. The crystals grow to typical dimensions of 0.2 × 0.4 × 0.6 mm.

absence of all $2n + 1$ reflections along the a^*, b^* and c^* axes. Thus, the $(His)_6$HPrK/P crystal takes the space group $P2_12_12_1$ and the unit-cell parameters are $a = 117.1$, $b = 127.7$, $c = 170.7$ Å, $\alpha = \beta = \gamma = 90.0^\circ$. On the basis of the unit-cell volume (2.564×10^6 Å^3) and the assumption of six monomers per asymmetric unit, the calculated V_M is 2.9 Å^3 Da^{-1} (Matthews, 1968). The presence of a hexamer is supported further by the results of the slow self-rotation function as implemented in *GLRF* (Tong & Rossmann, 1997) with resolution and radius cutoffs of 10–3.5 Å and 20 Å, respectively, which reveals a sixfold rotation axis at a peak height that is 8.6 above σ.

Searches for heavy-atom derivatives are under way, as are multiple-wavelength anomolous diffraction experiments using crystals of selenomethionine-substituted HPrK/P in complex with AMPPCP (Hendrickson, 1991).

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 473, the Fonds der Chemischen Industrie (to JS), NIH training grant AI07472 (to GSA) and NIH grant GM49244 (to RGB).

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