Study of the structure–activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis*

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In an attempt to investigate the molecular basis of pyrazinamide hydrolysis by the PncA protein from *Mycobacterium tuberculosis*, we determined the pyrazinamidase activity of nine PncA mutants bearing a single amino acid substitution. Among them, three mutants (D8G, K96T and S104R) had virtually no activity ($\leqslant$ 0.004 unit/mg), five (F13S, T61P, P69L, Y103S and A146V) retained a low level of activity (0.06–0.25 unit/mg) and one (T167L) exhibited a wild-type activity (1.51 units/mg). The possible structural effects of these substitutions were assessed by analysing a three-dimensional model of the PncA protein constructed on the basis of the crystal structure of the N-carbamoylsarcosine amidohydrolase (CSHase) from *Arthrobacter* sp., an amidohydrolase which was found by hydrophobic cluster analysis to be closely related to PncA. In the PncA model, five of the mutated residues, Asp-8, Phe-13, Lys-96, Tyr-103 and Ser-104, were located within a 6 Å sphere around the cysteine residue Cys-138, which could be the counterpart of the active cysteine residue Cys-177 found in the CSHase. Among the remaining mutated residues, Thr-61, Pro-69 and Ala-146 were found to be more distant from Cys-138 but were associated with structural elements contributing to the catalytic centre, whereas Thr-167 was situated in an α-helix located far from the putative active site. These data suggest that the decrease in pyrazinamidase activity observed in the PncA mutant proteins is well correlated with the structural modifications the mutations can cause in the environment of the putative active cysteine Cys-138.

Key words: amidohydrolase, homology modelling, hydrophobic cluster analysis, pyrazinamide.

INTRODUCTION

The anti-bacterial activity of pyrazinamide (PZA), one of the first-choice drugs in the chemotherapy of tuberculosis, requires an enzyme, pyrazinamidase (PZase), which converts PZA into pyrazinoic acid (POA). Recent reports have established clearly that mutations in the *pncA* gene encoding PZase lead to the loss of PncA activity and are the main mechanism of PZA resistance in *Mycobacterium tuberculosis* [1–7]. A large diversity and a wide distribution along the entire length of the *pncA* gene are the two main features of the resistance-associated mutations. However, we have reported recently that a significant proportion of the substitutions are clustered in three regions in the *M. tuberculosis pncA* gene [5]. At the protein level, these regions were found to be well conserved among the amino acid sequences of PncA proteins from different bacterial species. Moreover, they contain the amino acids that are aligned with the residues involved in the active site of the N-carbamoylsarcosine amidohydrolase (CSHase) from *Arthrobacter* sp., an enzyme exhibiting an amidohydrolytic function similar to that of PncA and belonging, as PncA, to the isochorismatase family [8,9]. Therefore, we speculated that such regions might be important components in the active site of the PncA protein and that mutations occurring in these regions could drastically affect the PZase activity.

In the present report, we confirm this hypothesis by investigating the functional and structural effects of *pncA* mutations characterized previously from PZA-resistant clinical strains of *M. tuberculosis* [5]. Nine mutant proteins were overexpressed, purified and their PZase activities measured and compared with that of the wild-type enzyme. The molecular basis of the loss of PZase activity in the mutants was investigated on the basis of a model structure of the wild-type PncA protein.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Nine clinical PncA mutants of *M. tuberculosis*, as well as the wild-type reference strain H37Rv and one PZA-susceptible *M. tuberculosis* clinical isolate, were studied. *Escherichia coli* Nova-blue and BL21(DE3) competent cells (Novagen) were used as host cells for pET-30 Ek/LIC (Novagen) plasmid transformation and for expression of hexahistidine-tagged PncA proteins (His_6-PncA), respectively. *E. coli* cells were grown in Luria–Bertani broth at 37 °C in the presence of kanamycin (30 μg/ml).

Cloning of the *M. tuberculosis* PZase gene (*pncA*) in *E. coli*

The *pncA* gene was amplified by PCR using oligonucleotides LIC1 (5′-GACGACGACAAGATGCGGGCGTTGATCATC-GT-3′) and LIC2 (5′-GAGGAGAAGCCCGGTTCAGGAGC-TGCAAACCAACT-3′) with ligation-independent cloning (LIC) sites (underlined bases) at their 5′ ends. *pncA* was subsequently inserted into compatible sites LIC in the expression vector pET-30 Ek/LIC, resulting in pET-30 Ek/LIC::His_6-PncA. Transformation of *E. coli* NovaBlue cells with plasmid pET-30 Ek/LIC::His_6-PncA was performed according to the manufacturer's instructions. Plasmid DNA was then extracted by the procedure of Birnboim and Doly [10] and was introduced in *E. coli* BL21(DE3) cells by using the Gene Pulser electroporator

Abbreviations used: PZA, pyrazinamide; POA, pyrazinoic acid; PZase, pyrazinamidase; CSHase, N-carbamoylsarcosine amidohydrolase; HCA, hydrophobic cluster analysis.

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(Bio-Rad, Ivry sur Seine, France), set at 2.5 kV, 200 Ω and 25 μF. The plasmid DNA extracted from the recombinant *E. coli* cells was systematically sequenced to ensure that the *pncA* gene is in-frame with the appropriate hexahistidine tag for expression of the His_6-PncA protein.

Purification of wild-type and mutant PncA proteins from *E. coli* transformants

Luria–Bertani broth (10 ml) containing kanamycin (30 μg/ml) was inoculated with a fresh colony of recombinant *E. coli* BL21(DE3)::His_6-PncA and grown at 37 °C to an absorbance of 0.5–1 at 600 nm. The inoculum was then transferred to 250 ml of the same medium in a 1000 ml shaking flask. When the culture reached an A_{600} value of 0.5–1, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM. After incubation for 6 h at 37 °C, cells were harvested by centrifugation at 5000 ***g*** for 5 min at 4 °C. The pellet was resuspended in 5 ml of binding buffer (5 mM imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9) and frozen at −80 °C. The cells were then thawed on ice, lysed by sonication using a microtip probe (Vibra Cell, Bioblock Scientific, power level set to 3–4 at 50 % duty for 20 bursts) and centrifuged at 39000 ***g*** for 20 min at 4 °C. The resulting supernatant corresponded to the soluble cytosolic fraction, while the pellet contained the insoluble fraction. The soluble fraction was filtered through a 0.45 μm membrane before being loaded on to a 2.5 ml His-bind column (Novagen). After one step of washing with a buffer containing 60 mM imidazole, 0.5 M NaCl and 20 mM Tris/HCl, pH 7.9, the bound proteins were eluted with elution buffer (1 M imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9). The fractions containing the recombinant PncA protein were analysed by SDS/PAGE. The protein concentration was determined with reference to a standard BSA scale analysed by SDS/PAGE with a computerized densitometer (Bio-Rad).

Enzyme assay

Purified His_6-PncA (10 μl) was added to 990 μl of a reaction mixture containing 20 mM Tris/HCl, pH 7.0, and 8 mM PZA. The mixture was incubated at 37 °C for 4 h. Ferrous ammonium sulphate (25 mM) was added. Precipitates were removed by centrifugation (13000 ***g*** for 10 min) and the change in A_{460} was measured spectrophotometrically. The value of A_{460} could be converted to equivalents of POA by referring to a standard curve prepared by treating various known concentrations of free POA by the same assay procedure, using an extinction coefficient $\Delta\epsilon_{460}$ of 6×10^{-4} $\mu M^{-1} \cdot cm^{-1}$. The amount of PZase enzyme required to produce 1 μmol of POA/h was defined as 1 unit. Specific activities were expressed as enzyme units/mg of total protein. The amount of purified His_6-PncA protein used in the reaction mixture was increased by 10-fold when the initial specific activity was lower than 0.05 unit/mg.

Hydrophobic cluster analysis (HCA) and modelling of the three-dimensional structure of *M. tuberculosis* wild-type PncA protein

The model of the three-dimensional structure of the wild-type PncA protein was constructed by homology modelling using the computer program Swiss-Model (htttp://www.expasy.ch/spdbv/mainpage.htm) with the optimized mode option allowing the user to improve manually the alignment between the template and the model sequence [11]. The experimental structure of the CSHase from *Arthrobacter* sp. (Protein DataBank entry 1NBA) was used as a template. The three-dimensional model obtained was checked with the programs Procheck and Verify3D [12,13].

The optimized alignment used in Swiss-Model was obtained using the bidimensional HCA method (DRAWHCA program, http://www.lmcp.jussieu.fr/~mornon), as described elsewhere [14,15]. Briefly, this bidimensional method is based on the one-dimensional sequence being represented on an α-helical net in which hydrophobic amino acids (Val, Ile, Leu, Met, Tyr, Phe and Trp) do not distribute randomly but form clusters, which have been shown to mainly correspond to the internal faces of regular secondary structures [16]. HCA thus allows one to favourably combine sequence comparison with secondary-structure prediction, a property that is useful to fit the best proposed alignment to the two-dimensional structure for low levels of sequence identity.

RESULTS

Purification of wild-type and mutant His_6-PncA proteins

Two wild-type and nine mutant PncA proteins were over-expressed in *E. coli* BL21(DE3) and purified. All the mutants harboured a single amino acid substitution located either in the conserved (D8G, F13S, P69L, K96T, Y103S, S104R and A146V) or non-conserved (T61P and T167I) regions identified previously in the *M. tuberculosis* PncA protein [5]. After induction by isopropyl β-D-thiogalactoside, the PncA proteins were expressed in *E. coli* BL21(DE3) to high levels corresponding to 20–30 % of the total protein content of the crude extracts. After purification on a His-bind column, the His_6-PncA proteins recovered were > 85 % purity (results not shown).

Activities of the recombinant His_6-PncA proteins

The proteins from the soluble cytosolic fractions were used for determining the specific activities of the PncA mutants (Table 1). The values obtained for the reference strain H37Rv and the clinical PZA-susceptible strain were comparable (1.33 and 4.43 units/mg of protein, respectively). In contrast, three groups of His_6-PncA mutants could be distinguished according to the values of their specific activities. The first group contained three mutants (K96T, D8G and S104R) displaying specific activities drastically reduced when compared with the wild-type PncA protein (⩽ 0.004 unit/mg, i.e. approx. 1000-fold lower than the wild-type PncA). The second group contained five mutants, F13S, Y103S, A146V, T61P and P69L, which retained a low level

Table 1 PZase activities of wild-type (WT) and PncA mutant proteins

AS, active site, defined by the residues located within a 6 Å sphere from the putative active Cys-138 found at the centre of the catalytic cleft (shown in Figure 2).

Strain	Specific activity (units/mg of protein)	Location of the mutated residue in the predicted PncA secondary structure
PncA WT*	1.33	–
PncA WT†	4.43	–
K96T	0.002	β3 (AS)
D8G	0.004	Loop connecting β1 and αC (AS)
S104R	0.004	Loop connecting β3 and αD (AS)
F13S	0.06	Loop connecting β1 and αC (AS)
Y103S	0.11	Loop connecting β3 and αD (AS)
A146V	0.17	αE
T61P	0.21	Loop connecting β2 and β3
P69L	0.25	Loop connecting β2 and β3
T167I	1.51	αF

* Reference strain H37Rv.
† PZA-susceptible clinical isolate.

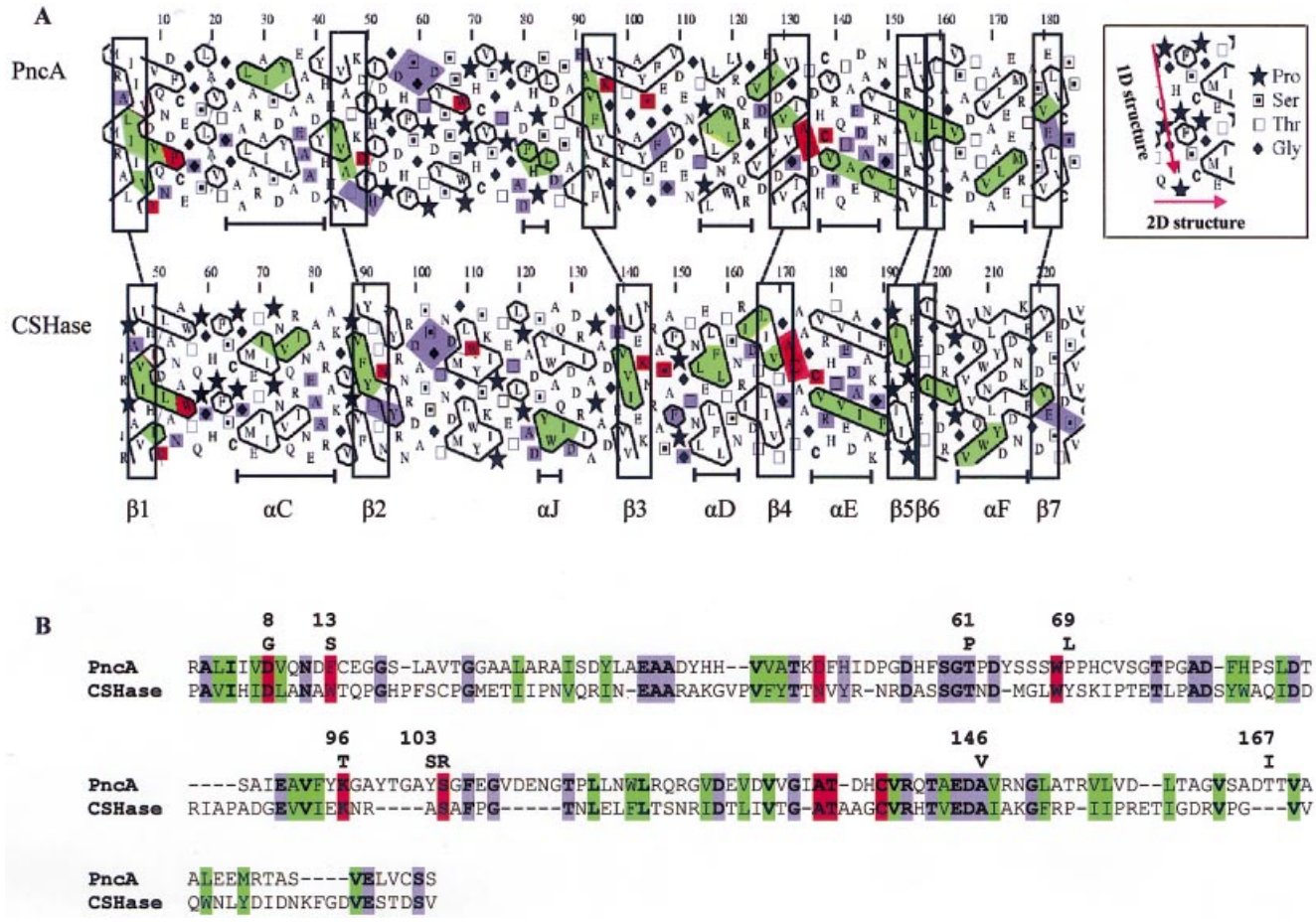

Figure 1 HCA plot comparison of the *M. tuberculosis* PncA (Arg-2–Ser-186) and *Arthrobacter* CSHase (Pro-45–Val-227) sequences (A) and deduced linear alignment (B)

(**A**) The one-letter code is used for all amino acids except for proline, glycine, threonine and serine residues for which special symbols are used, as indicated in the box to the right. The contours encircling the hydrophobic residues Val, Leu, Ile, Met, Phe, Tyr and Trp are automatically drawn by the program to form clusters of adjacent hydrophobic residues, a cluster generally corresponding to the internal face of a regular secondary structure [16]. Thus the segmentation of each protein into successive secondary-structure elements appears along the horizontal axis, while the one-dimensional amino acid sequence can be read on an oblique axis, as indicated by the two red arrows in the box. The hydrophobic residues conserved between the two protein sequences are coloured green. Alanine and histidine residues closely associated with hydrophobic clusters, which can substitute hydrophobic and aromatic residues [15,20], are also coloured green. Residues involved in the active site of the CSHase, and the corresponding residues in the putative active site of the *M. tuberculosis* PncA protein, are indicated in red. The other conserved residues found between the two proteins are coloured violet. The secondary-structure elements found in the three-dimensional structure of the CSHase are indicated under the CSHase plot and are reported on the PncA plot using vertical boxes (β-strands) and horizontal lines (α-helices). (**B**) Deduced linear alignment between the PncA protein and the CSHase. Identical amino acid residues are shown in bold. The amino acid substitutions investigated in the present study are indicated above the alignment, using the *M. tuberculosis* numbering system [1].

of activity, with values ranging from 0.06 to 0.25 unit/mg, i.e. approx. 20-fold lower than those found for the wild-type enzyme. Finally, in the third group was the mutant T167I, characterized by a specific activity (1.51 units/mg) unchanged with respect to the values found for the wild-type proteins.

HCA and modelling of the three-dimensional structure of the *M. tuberculosis* wild-type PncA protein

A model of the *M. tuberculosis* PncA protein was constructed by homology modelling on the basis of the known three-dimensional structure of the *Arthrobacter* CSHase, which was the most related amidohydrolase found in the Protein DataBank (26% identity, after optimized alignment, see below). Because the two proteins share a low sequence relatedness, the amino acid sequences of the CSHase and of PncA were analysed and compared using the bidimensional HCA method (Figure 1A), which allows detection of conserved secondary-structure elements in proteins exhibiting a low degree of sequence identity [14,15]. The 12 hydrophobic clusters found in the CSHase, which correspond to seven β-strands and five α-helices, are well conserved in PncA (common hydrophobic residues indicated in green on Figure 1A). Indeed, the alternating order of the β-strands and α-helices observed in the HCA plot of the *Arthrobacter* CSHase, represented by the succession of hydrophobic clusters Val-Ile-Ile (β1), Ile-Val-Ile (αC), Val-Phe-Tyr (β2), Tyr-Trp (αJ), Val-Val-Ile (β3), Leu-Phe-Leu (αD), Leu-Val (β4), Val-Val-Ile (αE), Ile-Ile-Ile ($\beta5+\beta6$), Val-Trp-Tyr (αF) and Val (β7), is well conserved in the PncA protein, where it corresponds to Leu-Ile-Val (β1), Leu-Ile-Tyr (αC), Val-Val-Ala (β2), Phe-His (αJ), Ala-Val-Phe (β3), Leu-Trp-Leu (αD), Val-Val (β4), Val-Ala-Val (αE), Val-Leu-Leu ($\beta5+\beta6$), Val-Leu-Met (αF) and Val (β7). It is worth noting that most of these conserved hydrophobic amino acids correspond, in the multiple alignment of the whole iso-

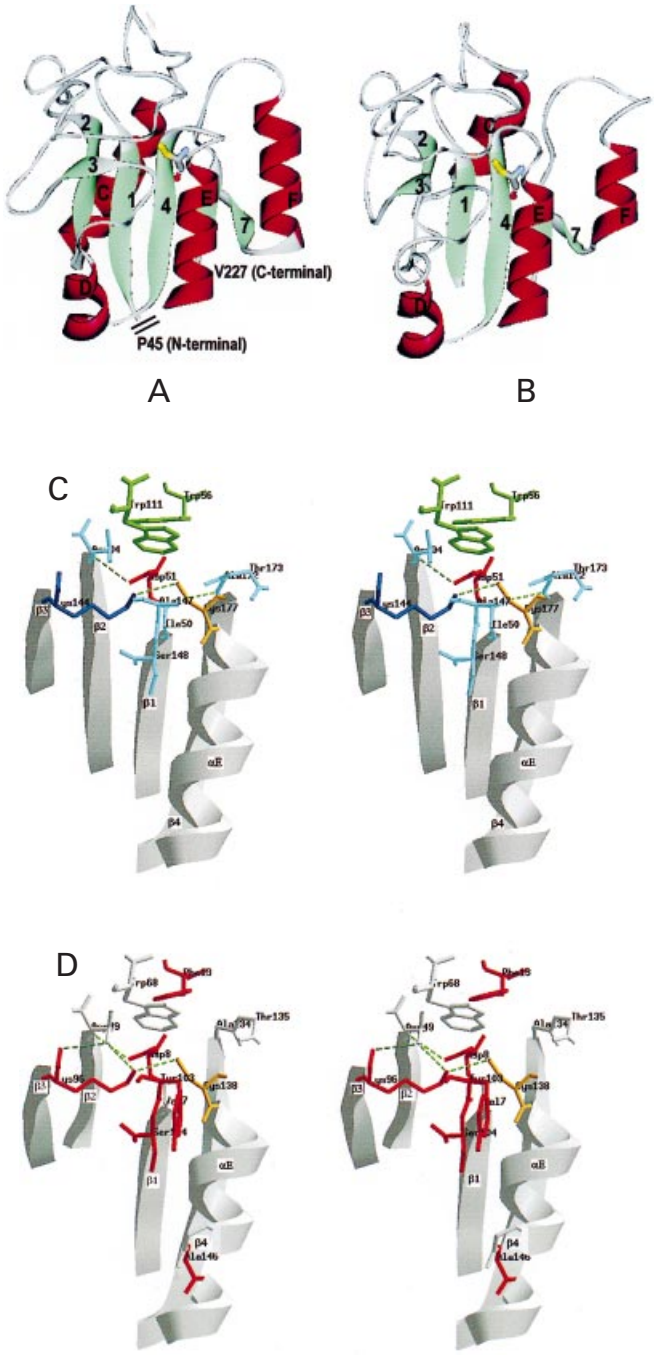

Figure 2 Three-dimensional representations of the *Arthrobacter* CSHase crystal structure (Pro-45–Val-227, A and C) and of the *M. tuberculosis* PncA protein model structure (B and D)

(**A** and **B**) Schematic drawings of the overall structure of the *Arthrobacter* CSHase subunit and the *M. tuberculosis* PncA protein, respectively. The active Cys-177 residue of the CSHase, and the corresponding Cys-138 in the PncA model, are shown using light blue (main chain) and yellow (side chain) sticks. The α-helices and the β-strands are labelled with letters and numbers, respectively, according to the labelling used for the secondary-structure elements in the CSHase structure [8]. (**C** and **D**) Stereoscopic views of the catalytic centres in the models of the *Arthrobacter* CSHase and the *M. tuberculosis* PncA protein, respectively. The Cα backbone and side chains of the residues of interest are represented by sticks. The α-helices and β-strands associated with these residues are drawn as grey ribbons. The hydrogen bonds are indicated by green dashed lines. In the CSHase catalytic centre (**C**), the nucleophile Cys-177, the acidic Asp-51, the basic Lys-144 and the aromatic Trp-56 and Trp-111 are yellow, red, blue and green, respectively. In the hypothetical catalytic centre of the PncA protein, the putative active Cys-138 is yellow, whereas the amino acid substitutions found in the clinical mutants analysed in the present study are in red.

chorismatase family, to positions always or most often occupied by hydrophobic residues, representing core residues that are decisive for the α/β fold adopted by these enzymes (results not shown). Moreover, most of the catalytic residues involved in the active site of the CSHase (Asp-51, Trp-56, Asn-94, Lys-144, Ala-172, Thr-173 and Cys-177, in red on Figure 1A), which are found closely associated with the hydrophobic clusters corresponding to strands β1, β2, β3 and β4 and to helix αE in the CSHase (Figure 1A), are highly conserved in PncA in which they are found in association with the hydrophobic clusters representing the corresponding putative secondary structures β1 (Asp-8 and Phe-13), β2 (Asp-49), β3 (Lys-96), β4 (Ala-134 and Thr-135) and αE (Cys-138). A total of 32 other amino acids (in violet on Figure 1A) were also found to be highly conserved and associated with identical hydrophobic clusters in both proteins.

Considering the hydrophobic clusters and the conserved residues as anchoring points, an optimized sequence alignment of PncA and the CSHase was produced from the HCA plots (Figure 1B) and was used for constructing by homology modelling a three-dimensional model of the *M. tuberculosis* PncA protein. The global fold of the model structure of PncA, as well as the corresponding part of the crystal structure of the *Arthrobacter* CSHase (between residues Pro-45 and Val-227), are presented in Figures 2(A) (CSHase) and 2(B) (PncA). Comparison of the two models indicated that 167 Cα carbons can be easily superimposed with a root mean square deviation of 2.0 Å. Moreover, analysis of the Ramachadran plot of the PncA model revealed that 97% of the amino acids are located in the most favoured regions or in the additionally allowed regions, with only 2% of the residues in the generously allowed regions, indicating a reasonable conformation for most of the Cα included in the PncA model. Finally, the three/one-dimensional scores of our model, determined using the Verify 3D algorithm [12], were always positive and similar to those obtained with the template structure 1NBA.

Analysis of the model structure of the *M. tuberculosis* PncA protein

In the PncA model, the putative catalytic centre (Figure 2D) would be located in a pocket formed by one α-helix (αE) and four β-strands (β1, β2, β3 and β4). In this pocket, the conserved active cysteine residue Cys-138, which corresponds to Cys-177 in the CSHase, is located close to the conserved residues Asp-8, Trp-68, Lys-96, Ser-104, Ala-134 and Thr-135, which are all found in the active site of the CSHase (Asp-51, Trp-111, Lys-144, Ser-148, Ala-172 and Thr-173, respectively; Figures 1B and 2C). Moreover, the aromatic residue Phe-13 and the acidic residue Asp-49, also found in the active-site region of the PncA model (Figure 2D), are predicted to correspond to Trp-56 and Asn-94 in the active site of the CSHase, respectively. By contrast, the aromatic residue Tyr-103, located close to Cys-138 in the PncA model, seems to have no counterpart in the CSHase, where it corresponds to residue Ala-147. In the PncA model, the side chains of the two residues Asp-8 and Lys-96 would point towards the active-site cysteine Cys-138, as do the equivalent residues Asp-51 and Lys-144 towards Cys-177 in the CSHase (Figures 2C and 2D). Finally, it must be noted here that the hydrogen bond found in the CSHase active site between the active Cys-177 and the basic residue Lys-144, which was suggested to increase the nucleophilicity of Cys-177 [8], would be conserved in the PncA active-site model between the corresponding residues Cys-138 and Lys-96.

Strikingly, five of the nine mutants (D8G, F13S, K96T, Y103S and S104R) analysed in the present study harboured a sub-

stitution affecting a residue located at the vicinity (less than 6 Å) of the putative active Cys-138 (Figure 2D). The four remaining mutants, T61P, P69L, A146V and T167I, were characterized by the replacement of amino acid residues more distant from Cys-138, but three of them were altered in positions associated with structural elements contributing to the active site, i.e. Ala-146 in the αE helix (Figure 2D) and Thr-61 and Pro-69 in the long loop connecting strands β2 and β3 and holding Trp-68 (Figures 1A and 1B).

DISCUSSION

By determining the activity of the nine PncA mutants included in the present study, we have shown that the modification of the amino acid residues Asp-8, Lys-96 and Ser-104 in the mutants D8G, K96T and S104R resulted in enzymes showing specific activities drastically impaired ($\leqslant 0.004$ unit/mg), thus suggesting that these residues are essential for the PncA activity. Accordingly, the three residues are located close to the putative active cysteine residue Cys-138 in the three-dimensional model of PncA (Figure 2D), i.e. in spatial positions similar to those occupied by the corresponding residues Asp-51, Lys-144 and Ser-148 around the active cysteine Cys-177 in the CSHase (Figure 2C) [8,17]. Therefore, Asp-8, Lys-96 and Ser-104 may play a role in the PncA catalytic centre similar to that attributed to Asp-51, Lys-144 and Ser-148 in the CSHase, a hypothesis confirmed by the fact that these residues have been demonstrated previously to be strictly conserved in the CSHase and in PncA proteins from different bacterial species [5]. It has been suggested from the crystal structure of the CSHase that Asp-51 and Lys-144 are important for the activity of this amidohydrolase because (i) the two residues interact via a salt bonding and (ii) Lys-144 forms with Cys-177 a hydrogen bond that may increase the nucleophilicity of the catalytic cysteine residue [8]. In the model of PncA, the replacement of the corresponding residues Lys-96 and Asp-8 by neutral threonine and glycine residues, respectively, could reduce the nucleophilicity of the putative active cysteine Cys-138 and would interrupt the hydrogen bond connecting Lys-96 to Cys-138. Regarding the serine found in position 104 in PncA, substitution of this residue by an arginine reduced the PZase activity to 0.1% of the wild-type activity. The corresponding Ser-148 found in the CSHase has not been attributed a specific role in the active site of this enzyme [8]. However, the replacement of Ser-104 by an arginine in the CSHase active site, as well as in PncA, leads to steric hindrance between the bulkier side chain of arginine and the neighbouring residues located in the αE helix holding Cys-138 (Figures 2C and 2D). The steric constraints resulting from such interactions could significantly alter the folding of the mutant protein and/or the positioning of the side chain of the catalytic cysteine residue, readily explaining the loss of PncA activity seen in the S104R mutant.

The five mutants F13S, Y103S, A146V, T61P and P69L exhibited a significant decrease in PncA activity (see Table 1). In the PncA model, Phe-13 is located on the β1–αC loop close to Cys-138 (Figure 2D). It has been reported that a phenylalanine residue is strictly conserved in position 13 in all the PncA proteins that have been sequenced to date [5], supporting the idea that this residue may be crucial for the PncA activity. Accordingly, in the crystal structure of the CSHase, the Trp residue found in position 56, which corresponds to Phe-13 in PncA, is involved with Trp-111 in the hydrophobic cleft surrounding Cys-177 (Figure 2C). This hydrophobic environment is known to be important for the binding of the substrate [8,17]. In PncA, it is likely that Phe-13 also contributes with Trp-68 (the counterpart of Trp-111 found in the CSHase) to form a cluster of hydrophobic aromatic residues in the vicinity of the active cysteine Cys-138 (Figure 2D). In the F13S mutant, replacement of Phe-13 by a serine may significantly reduce the hydrophobic environment required for the binding of the substrate and would account for the decrease in PncA activity observed for the mutant protein.

Among the putative catalytic residues forming the PncA active site, Tyr-103 is the sole residue to have no equivalent in the active site of the CSHase, so that its role in the putative active site of PncA is less clear. Nevertheless, this aromatic residue is found on the β3–αD loop that faces helix αE, holding the essential catalytic residue Cys-138 (Figure 2D). Therefore, the substitution of Tyr-103 by a serine, as found in the mutant Y103S, may have a significant impact on helix αE and, consequently, on the positioning and/or the environment of the side chain of Cys-138, resulting in a 10–40-fold decrease in catalytic activity.

The moderate level of activity (0.1–0.2 unit/mg) displayed by the three remaining mutants T61P, P69L and A146V seems to be correlated, in the putative PncA structure, to the location of the corresponding substituted amino acids, which are relatively distant from the active cysteine. Residue Ala-146, which is approx. 9 Å distant from the centre of the catalytic cleft, would be located in the C-terminal part of the αE helix, which holds in its N-terminal part the active cysteine residue of PncA (Cys-138). The replacement of Ala-146 by a bulkier valine residue could therefore disturb the conformation and/or the folding of the αE helix, thereby decreasing significantly the PZase activity in the mutant protein. With regard to residues Thr-61 and Pro-69, they would both be located in a loop that does not participate directly in the catalytic centre of PncA (results not shown). However, it is noteworthy that Thr-61 and Pro-69 are located close to Trp-68, which is one of the conserved residues found in the PncA active site (Figure 2D). In addition, the two mutants T61P and P69L are characterized by substitutions involving a proline, a residue that is known to produce bends in regular secondary structures. Therefore, it can be hypothesized that the 5–20-fold decrease in activity observed in the mutants T61P and P69L results from a more global change in the conformation of the PncA protein.

The mutant T167I exhibited a wild-type level of activity (1.51 units/mg). Accordingly, Thr-167 is located in the C-terminal part of the PncA protein, in an α-helix predicted to superimpose in the CSHase on to helix αF located approx. 16 Å apart from the active-site cysteine residue (Figures 2A and 2B). Therefore, the substitution of Thr-167 by Ile in PncA probably occurs too far away from the active site to have a significant effect on the PncA activity. The existence of PZA-resistant strains of *M. tuberculosis* harbouring a *pncA* mutation but retaining a PZase activity has been reported previously [7]. Recently, new mechanisms of resistance to PZA linked to high active efflux for POA and lack of uptake for PZA have been identified in strains of *M. kansasii* and *M. smegmatis* naturally resistant to PZA [18,19]. Such mechanisms could explain the PZA resistance observed in the *M. tuberculosis* strain producing the T167I mutant, which is characterized by a wild-type level of PncA activity.

In conclusion, the present study provides the first structural and kinetic insights for the *M. tuberculosis* PncA protein. The kinetic study and the three-dimensional modelling of PncA and the nine mutants presented in this report strongly suggest that the amino acids found at positions 8, 13, 61, 69, 96, 103, 104 and 146 are functionally and/or structurally important in PncA. In addition, the level of activity displayed by the PncA mutants seems to be well correlated with the location of the mutated residues and the structural modifications they can cause in the vicinity of the putative active-site cysteine residue found at position 138. However, further structural studies are required to

validate the positioning suggested by the PncA model for these catalytically important amino acid residues, as well as to elucidate the catalytic mechanism of PZA hydrolysis by PncA.

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Received 17 August 2000/18 September 2000; accepted 9 November 2000