Evidence that cysteine-166 is the active-site nucleophile of *Pseudomonas aeruginosa* amidase: crystallization and preliminary X-ray diffraction analysis of the enzyme

Sebastien FARNAUD*, Renée TATA*, Maninder K. SOHI†, Tommy WAN†, Paul R. BROWN*[1] and Brian J. SUTTON†

*Molecular Biology and Biophysics Section, Division of Biomolecular Sciences, King's College London, Strand, London WC2R 2LS, U.K., and
†The Randall Institute, King's College London, 26–29 Drury Lane, London WC2B 5RS, U.K.

Wild-type and site-specific mutants C166S and C166A (Cys-166 → Ser and Cys-166 → Ala respectively) of the amidase (acylamide amidohydrolase, EC 3.5.1.4) from *Pseudomonas aeruginosa* were expressed in *Escherichia coli* by using the vector pKK223-3. Both mutant proteins were catalytically inactive but showed complete cross-reactivity with polyclonal antiserum raised against the wild-type enzyme, as well as CD spectra identical with that of the wild-type enzyme, which were indicative of correct folding. Cys-166 is therefore implicated as the active-site nucleophile. Titration of free thiol groups with 5,5′-dithiobis-(2-nitrobenzoic acid) indicated that Cys-166 is not a rapidly reacting residue. Crystals of both wild-type and C166S amidase grew with identical, rhombohedral morphology; X-ray diffraction analysis established the unit cell dimensions ($a = b = c = 84$ Å; $\alpha = \beta = \gamma = 75$ °) and space group (*R*3 or *R*32). These results imply a quaternary structure of six subunits, with most probably 32 symmetry; the existence of a hexameric structure was supported by molecular mass determinations based on gel filtration and electrophoretic mobility.

Key words: quaternary structure, site-directed mutagenesis, thiol groups.

INTRODUCTION

The inducible aliphatic amidase (acylamide amidohydrolase, EC 3.5.1.4) encoded by the *amiE* gene of *Pseudomonas aeruginosa* catalyses the hydrolysis of a small range of aliphatic amides, but its substrate specificity can be changed through point mutations and extended to longer aliphatic amides [1] and aromatic amides including phenylacetamide [2] and acetanilide (*N*-phenylacetamide) [3]. The enzyme belongs to a group of amidases with similar properties, including those from *Methylophilus methylotrophus* [4], *Arthrobacter* sp. J-1 [5], *Rhodococcus* sp. [6] and *Helicobacter pylori* [7]. The amino acid sequence of the *Pseudomonas* enzyme [8,9] shows 80 % identity and 90 % similarity with the *Rhodococcus* enzyme, and 75 % identity and 87 % similarity with the *Helicobacter* enzyme. Despite their similarities, the *Rhodococcus*, *Pseudomonas* and *Arthrobacter* enzymes have been reported as tetrameric, hexameric and octameric respectively. The amidase group is also related by sequence similarity to the nitrilase/cyanide hydratase enzyme family [10,11] and, because a cysteine residue is the active-site nucleophile for this group, Cys-166 was predicted to have a similar function for amidase, interacting with the carbonyl group of the substrate amide to form a transitory acyl complex (as outlined by Jallageas et al. [12]). However, no direct evidence has been obtained that implicates this residue in the catalytic cycle. No three-dimensional structures have been reported either for the amidase group or for the related nitrilase family.

MATERIALS AND METHODS

Site-directed mutagenesis

The method used is described in detail in the Promega manual (altered sites I *in vitro* mutagenesis system). The *amiE* gene was amplified by PCR by using primers with *Eco*RI sites at their 5′ ends. The PCR product was cut with *Eco*RI and ligated into pAlter-1 (Promega). The orientation of the gene was determined by digestion with *Pst*I, for which there is an asymmetrically placed site in the *amiE* gene. Single-stranded recombinant pAlter-1 DNA was generated by using helper phage R408.

Oligonucleotides (purchased from Pharmacia) 5′-GATCAGCCTGATTATCTCCGACGACCCCACT-3′ and 5′-GATCAGCCTGATTATCGCCGACGACCCCACT-3′, incorporating a silent change (single T) that eliminated a unique *Bcl*I site in the *amiE* gene, were used to introduce the mutations C166S and C166A (TGC → TCC, GCC) respectively. Plasmids were analysed for incorporation of the mutagenic oligonucleotides after growth in *Escherichia coli* strain Gm48 (*dam*$^-$) by digestion with *Bcl*I. Those that were not cleaved were sequenced to confirm that the mutations had been incorporated.

Expression of *amiE* genes, and enzyme assays

Mutated and non-mutated *amiE* genes were excised with *Eco*RI from pAlter-1 and cloned into pKK223-3 (Pharmacia). Recombinant plasmids were transformed into *Escherichia coli* strain JM109; for enzyme production, transformants were grown overnight in Luria–Bertani medium containing 1 mM isopropyl β-D-thiogalactoside and 125 μg/ml ampicillin. Extracts were prepared from cells grown overnight at 37 °C in 5 ml of Luria–Bertani medium. After pelleted cells had been resuspended in saline, they were re-centrifuged and resuspended in 0.5 ml of 50 mM Tris buffer, pH 7.2, containing 1 mM EDTA and 0.5 mM dithiothreitol. After sonication for 30 s at 0 °C in an MSE Mullard ultrasonic disintegrator (power output 50 W), cell debris was removed by centrifugation at 12000 ***g*** for 15 min at 4 °C.

Abbreviation used: Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

[1] To whom correspondence should be addressed. Present address: Anatomy Department, King's College London, Strand, London WC2R 2LS, U.K. (e-mail paul.brown@kcl.ac.uk).

© 1999 Biochemical Society

Amidase assays were performed with both the acyltransferase reaction [13] and the glutamate dehydrogenase-coupled hydrolase reaction [14], with acetamide as substrate.

Amidase purification

Mutant and wild-type amidases were expressed in *E. coli* and purified as described previously [15], except that Sepharose S-400 was substituted for Sephadex G200 in the final step. After purification, the enzymes were concentrated by pressure filtration to 25 mg/ml in 50 mM Tris buffer, pH 7.2, containing 5 mM dithiothreitol. The degree of apparent heterogeneity of purified enzymes was estimated after electrophoresis by scanning Coomassie Blue-stained native polyacrylamide gels with an Agfa Studio Scanner II.

Amidase concentrations were determined from the specific absorption coefficient, $A^{1\%}_{1\,\mathrm{cm},280} = 1.37\ \mathrm{litre \cdot g^{-1} \cdot cm^{-1}}$ [14].

Immunodiffusion

Rabbit antiserum against amidase was prepared and used in double-diffusion experiments as described previously [16].

Reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2)

Reactions of purified amidases with Nbs_2 were performed as described previously [17].

CD analysis

Measurements were made with a Jobin Yvon CD6 spectrophotometer at 20 °C with the use of a cell with a 0.2 mm path length; the instrument was calibrated with epi-andosterone. For calculation of the mean residue ellipticity (θ), the mean residue mass for amidase was taken as 111.1 Da. CD spectra were recorded at protein concentrations of 1.2–1.5 mg/ml in 50 mM potassium phosphate buffer (pH 8.0)/0.1 mM EDTA.

Molecular-mass determination by gel filtration and electrophoresis

Elution volumes (V_e) for amidase and protein standards were determined on Pharmacia columns K26/1000 and XK26/70 containing Sephacryl S300HR and Sephacryl S400HR. Void volumes (V_0) were determined with Blue Dextran. Protein standards used on Sephacryl S300HR were thyroglobulin (669 kDa), catalase (232 kDa), amylase (200 kDa) and alcohol dehydrogenase (150 kDa). On Sephacryl S400HR, apoferritin (440 kDa) and carbonic anhydrase (29 kDa) were used in addition to thyroglobulin, amylase and alcohol dehydrogenase. Columns were eluted at 84 ml/h and 4 °C.

The electrophoretic mobilities of amidase and a selection of other proteins of known molecular mass were compared on a series of native polyacrylamide gels with different percentages of polyacrylamide (4%, 6%, 8% and 10%, w/v) as described by Hedrick and Smith [18]. The molecular mass of amidase was obtained from the linear relationship between retardation coefficients (K_r) and protein molecular mass. K_r values were obtained as slopes from Ferguson plots (semilogarithmic plots of R_F against polyacrylamide concentration, where R_F is the distance moved by the protein divided by the distance moved by the dye front).

Crystallization

Crystallization trials were performed by using the hanging-drop technique at both 16 and 4 °C in 24-well tissue culture plates (Flow Laboratories) with siliconized cover slips. Each drop consisted of 5 μl of the protein solution at 25 mg/ml mixed with an equal volume of the reservoir solution (50 mM Tris/5 mM dithiothreitol/1 mM EDTA) over a range of pH values with various precipitants. Drops were left to equilibrate with 0.5 ml of reservoir solution. The largest crystals grew with 15% (w/v) polyethylene glycol 4000 and 5%-satd. $(NH_4)_2SO_4$ as precipitant, at pH 7.0 or 7.2, over 2–3 weeks at 4 °C.

X-ray analysis

Crystals were mounted in thin-walled quartz glass capillary tubes, sealed with mother liquor enclosed and cooled to 5 °C. Diffraction data were recorded on an R-AXISII image plate mounted on a Rigaku RU200HB rotating-anode X-ray generator (Cu K_α; $\lambda = 1.5418$ Å) with focusing mirrors. Data were processed with DENZO [19].

RESULTS

Properties of C166S and C166A amidases

Grown under inducing conditions, *E. coli* harbouring recombinant pKK233-3 incorporating the wild-type *amiE* gene expressed high levels of enzyme activity, with amidase constituting approx. 7% of the total cell protein. In contrast, no amidase activity was detected under the same conditions in strains that harboured plasmids incorporating the C166S and C166A amidase genes. An examination of cell extracts from the three strains by PAGE showed the presence in each of a dominant band; on SDS/PAGE this band corresponded in molecular mass to the amidase monomer (results not shown). In immunodiffusion experiments the polyclonal rabbit antiserum raised against wild-type amidase revealed structural differences between the wild-type amidase and mutant amidases in which substrate specificity had been altered through single point mutations, as described previously [1]. However, the proteins produced in the two strains synthesizing mutant amidases C166S and C166A cross-reacted completely with wild-type amidase, with no detectable spurring (Figure 1). The two mutant amidases were tracked through the purification procedure by immunodiffusion: both enzymes displayed the same elution profiles as that of the wild-type enzyme. All purified amidases showed less than 3% con-

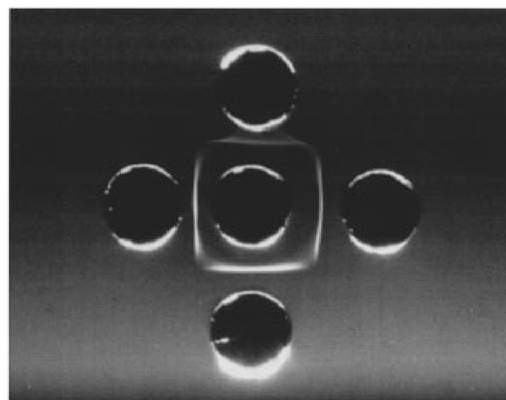

Figure 1 Precipitin reaction of mutant amidases with amidase antiserum

The centre well contained 10 μl of rabbit anti-amidase antiserum; the outer wells (clockwise from top left) contained extracts from *E. coli* expressing: mutant amidase (C166A), wild-type amidase, mutant amidase (C166S) and wild-type amidase.

© 1999 Biochemical Society

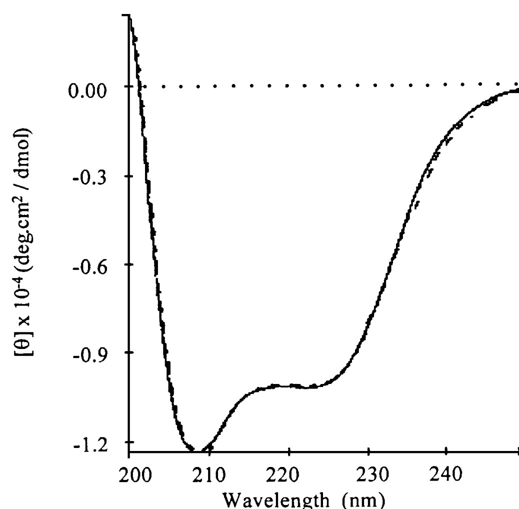

Figure 2 CD spectra of mutant and wild-type amidases

Spectra of wild-type amidase (solid line) and C166S mutant amidase (broken line) are shown. Enzyme concentrations were 1.3 mg/ml in 50 mM potassium phosphate buffer, pH 8.0.

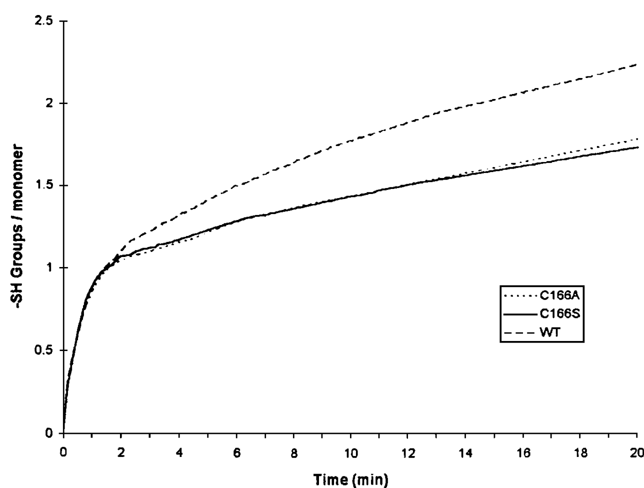

Figure 3 Comparison of reactions of purified amidases with Nbs_2

Nbs_2 (0.4 mM) was added to the purified wild-type (broken line) and mutant amidases C166A (dotted line) and C166S (solid line) (0.73 mg/ml) in 50 mM potassium phosphate (pH 8.0)/1 mM EDTA at 37 °C. The number of reacting thiol groups was calculated from the molar absorption coefficient, 14150 litre·mol^{-1}·cm^{-1}, for the Nbs^{2-} anion.

tamination with other proteins. No activity was detected in assays with 0.5 mg of purified mutant enzymes.

CD spectra for the three purified amidases were compared; Figure 2 shows that the CD spectra of the mutant and wild-type proteins were superimposable. We conclude that the mutant proteins adopted the native conformation.

Reactions between Nbs_2 and free thiol groups in purified wild-type and mutant amidases were compared by following the formation of nitrothiobenzoate at 412 nm (Figure 3). No significant differences were observed during the initial rapid phase of the reactions, but thereafter the reaction curves for the mutants diverged from that for the wild-type protein.

Figure 4 Crystals of wild-type amidase

The rhombohedral crystals, 0.1 mm in each dimension, were birefringent and were photographed under polarized light. Crystals of C166S amidase were identical in appearance.

Amidase molecular mass

Gel filtration on Sephacryl S300HR yielded a molecular mass of 221000 ± 11050 Da; the use of Sephacryl S400HR gave a molecular mass of 224000 ± 11200 Da (results not shown). Electrophoretic mobilities determined on PAGE gels gave a molecular mass of 215000 ± 8600 Da (results not shown).

Because the amidase subunit relative molecular mass, deduced from the primary structure [8], is 38441 Da, the number of monomers per molecule is clearly six.

Preliminary X-ray analysis of the crystals

The crystals of both wild-type and C166S mutant amidase grew with a rhombohedral morphology to approx. 0.1 mm in all dimensions (Figure 4). X-ray analysis of the capillary-mounted crystals at 5 °C caused radiation damage, and initial diffraction to better than 3 Å resolution progressively decayed to approx. 4.5 Å after only 10–15 frames (exposure time 45 min per 2 ° oscillation image). However, sufficient data were collected to allow indexing, which indicated a primitive rhombohedral lattice and space group either $R3$ or $R32$, with cell dimensions $a = b = c = 84$ Å and $\alpha = \beta = \gamma = 75$ °.

DISCUSSION

Cys-166 has been predicted to be the active-site nucleophile of amidase [11]; the total lack of activity of either of the mutant amidases C166S and C166A provides the first experimental evidence that this is a critical residue. The complete cross-reactivity of both mutants with polyclonal rabbit antisera raised against the wild-type enzyme reported here indicates that the mutants are both correctly folded; the identical CD spectra for all three proteins are evidence that no change in secondary structure has occurred as a result of the mutations. The fact that both wild-type and C166S mutant amidase crystals are isomorphous indicates that this mutation does not affect the quaternary structure or molecular packing.

It was proposed that the requirement of amidase for a reducing agent to maintain the enyzme in an active form was due to the susceptibility of a putative rapidly reacting thiol group to

© 1999 Biochemical Society

oxidation [20]. In the biphasic reaction of amidase with Nbs_2 the first, rapid, phase corresponds to the reaction of one thiol group per amidase monomer [17]. The results reported here show that the mutant amidases C166S and C166A are indistinguishable from the wild-type enzyme in the rapid phase of the reaction with Nbs_2. We therefore conclude that Cys-166 is not the rapidly reacting group and is probably not the group requiring the presence of a reducing reagent for the maintenance of enzyme activity. Interestingly, Kobayashi et al. [21] concluded, from its lethargic reactivity, that the active-site cysteine residue of the aliphatic nitrilase from *Rhodococcus rhodochrous* K22 was buried. The identity of the reactive cysteine residue of amidase is being investigated through a systematic programme of replacing the cysteine residues in the enzyme one by one. The motivation for this study is the need to facilitate the crystallization and the growth of crystals large enough for a high-resolution X-ray structure determination, processes that might be hampered by protein heterogeneity resulting from the enzyme's susceptibility to oxidation.

We report the first crystals of amidase and their preliminary X-ray analysis. Assuming a typical value for the packing density of the crystal, $V_m = 2.4$ Å^3/Da [22], if the space group is *R*3 each asymmetric unit contains two molecules of molecular mass approx. 38 kDa; if it is *R*32 each asymmetric unit contains one such molecule. The unit cell therefore contains either three dimers of a 38 kDa subunit (probably with non-crystallographic twofold symmetry) related by a crystallographic threefold axis (if *R*3), or six subunits of this molecular mass with a crystallographic threefold axis, and three orthogonal twofold axes (if *R*32). The data therefore indicate the presence of a hexamer, with certainly threefold symmetry and most probably 32 symmetry. Although the amidase molecule was previously reported to be hexameric, this conclusion was based on what now seems to have been an underestimate of the molecular mass, 201200 ± 1500 Da, derived from sedimentation equilibrium data, combined with an approximate value, 33000 Da, for the subunit molecular mass [20]. Furthermore, a tetrameric structure was implied by other results [23]. We therefore also report here further experimental determinations of the molecular mass of the enzyme that confirm its hexameric nature.

Crystallization trials of other cysteine mutants of amidase are continuing, as are attempts to increase the size of the present crystals. If flash-freezing to very low temperature can also be achieved, to overcome radiation damage, the crystal form reported here might yet be suitable for X-ray structure determination.

We thank Walter Gratzer (Randall Institute, London, U.K.) for help and advice with the CD measurements. The Wellcome Trust funded the installation of the X-ray focusing mirror system.

REFERENCES

1 Brown, J. E., Brown, P. R. and Clarke, P. H. (1969) J. Gen. Microbiol. **57**, 273–285
2 Betz, J. L. and Clarke, P. H. (1972) J. Gen. Microbiol. **73**, 161–174
3 Brown, P. R. and Clarke, P. H. (1972) J. Gen. Microbiol. **70**, 287–298
4 Silman, N., Carver, M. A. and Jones, C. W. (1991) J. Gen. Microbiol. **137**, 169–178
5 Asano, Y., Tachibana, M., Tani, Y. and Yamada, H. (1982) Agric. Biol. Chem. **46**, 1175–1181
6 Maestracci, M., Thiery, A., Arnaud, A. and Galzy, P. (1984) Arch. Microbiol. **138**, 315–320
7 Skouloubris, S., Labigne, A. and de Reuse, H. (1997) Mol. Microbiol. **25**, 989–998
8 Brammar, W. J., Charles, I. G., Hatfield, M., Cheng-pin, L., Drew, R. E. and Clarke, P. H. (1987) FEBS Lett. **215**, 291–294
9 Ambler, R. P., Auffret, A. D. and Clarke, P. H. (1987) FEBS Lett. **215**, 285–290
10 Bork, P. and Koonin, E. (1994) Protein Sci. **3**, 1344–1346
11 Novo, C., Tata, R., Clemente, A. and Brown, P. R. (1995) FEBS Lett. **367**, 275–279
12 Jallageas, J.-C., Arnaud, A. and Galzy, P. (1978) J. Gen. Appl. Microbiol. **24**, 103–114
13 Brammar, W. J. and Clarke, P. H. (1964) J. Gen. Microbiol. **37**, 307–319
14 Gregoriou, M. and Brown, P. R. (1979) Eur. J. Biochem. **96**, 101–108
15 Gregoriou, M., Brown, P. R. and Tata, R. (1977) J. Bacteriol. **132**, 377–384
16 Brown, P. R., Gregoriou, M. and Tata, R. (1978) Mol. Gen. Genet. **165**, 213–219
17 Tata, R., Marsh, P. and Brown, P. R. (1994) Biochim. Biophys. Acta **1205**, 139–145
18 Hedrick, J. L. and Smith, A. J. (1968) Arch. Biochem. Biophys. **126**, 155–164
19 Otwinowski, Z. (1993) in Data Collection and Processing (Sawyer, L., Isaacs, N. and Bailey, S., eds.), pp. 56–62, Daresbury Laboratory, Warrington
20 Brown, P. R., Smyth, M. J., Clarke, P. H. and Rosemeyer, M. (1973) Eur. J. Biochem. **34**, 177–187
21 Kobayashi, M., Yamada, N., Nagasawa, T. and Yamada, H. (1992) Biochemistry **31**, 9000–9007
22 Matthews, B. W. (1968) J. Mol. Biol. **33**, 491–497
23 Domingos, A., Karmali, A. and Brown, P. R. (1989) Biochimie **71**, 1179–1184

Received 17 November 1998/25 March 1999; accepted 12 April 1999

© 1999 Biochemical Society