Structural adaptation to selective pressure for altered ligand specificity in the Pseudomonas aeruginosa amide receptor, AmiC

Introduction

AmiC is the negative regulatory component of a ‘molecular switch’ which controls amide-inducible expression of the amidase operon of Pseudomonas aeruginosa (Wilson and Drew, 1991). Functionally, AmiC controls the activity of a transcription anti-termination factor, AmiR, which is required for transcription of the amidase operon past a ρ-independent terminator occurring downstream of the constitutive amidase promoter (Drew and Lowe, 1989). In the presence of an inducing amide, inhibition of AmiR by AmiC is relieved, and expression of amidase and other genes of the operon (including amiC and amiR) is induced. Sensitivity to amides in vivo is conferred by AmiC, which functions as a cytoplasmic amide receptor, and has been shown to bind acetamide with micromolar affinity in vitro (Wilson et al., 1993).

Structurally, AmiC is a two-domain protein related to periplasmic small-molecule binding proteins (SMBPs) such as the Escherichia coli branched amino acid binding protein, LivJ (Sack et al., 1989). The crystal structure of an AmiC–acetamide complex determined at 2.1 Å resolution, precisely defined the amide binding site in AmiC (Pearl et al., 1994), which lies at the interface of the two domains, which are stabilized in a ‘closed’ conformation by contacts with the bound ligand. This closed-down acetamide-bound form of AmiC represents the ‘on’ configuration of the regulatory system, in which AmiR is free to interact with the leader RNA of the operon transcript, and permit full expression. The mechanism of amide-switched regulation of AmiR by AmiC remains to be determined, but it probably involves disruption of a silencing AmiC–AmiR complex on binding of inducing amides to AmiC (Wilson et al., 1996).

Amidase expression in the PAC1 strain of Paeruginosa is strongly induced by acetamide (one carbon chain) and lactamide or propionamide (two carbon chain) but not by butyramide (three carbon chain), which instead acts as an anti-inducer in vivo and competes for binding to AmiC in vitro. Thus, lengthening of the aliphatic chain by a single carbon converts a strong agonist into an antagonist. We have now cloned and sequenced AmiC from the PAC181 strain of Paeruginosa, which was generated by classical in vitro selection for its ability to induce amidase expression in the presence of butyramide (Turberville and Clarke, 1981). The crystal structure of the AmiC protein from this mutant strain reveals the subtle structural adaptation that enables the butyramide-inducible phenotype, again demonstrating the remarkable adaptability of the ‘small molecule binding protein’ fold to the specific recognition of ligands.

Material and methods

Cloning and characterization of PAC181 amidase regulatory genes

The genes amiC and amiR were amplified by PCR from PAC181 chromosomal DNA using the oligonucleotides CRA (ATCCGATTTCACAGGAGAAACGGATG) and CRL (CCGATGCGAACGGCCATGACGATACCCCTTT), (Genosys, Cambridge, UK) and Taq DNA polymerase. The amplified DNA fragment was isolated from a preparative agarose gel, cloned initially into pUC19 (pSW181) and characterized by restriction enzyme mapping. The 1.8 kb amiCR fragment was then subcloned into the broad host range vector pMBB66EH (Temple et al., 1990) (plasmid pSW1181) and mobilized from E.coli S17-1 into Paeruginosa PAC327 (amiC+, amiR) via conjugation from E.coli Tac181 (amiC, amiR). Amidase activity was determined in intact cells under non-inducing, inducing and repressing conditions as described previously (Drew, 1984) and the results presented are the mean
Crystallization of PAC181-AmiC–butyramide complex

Protein expression and purification

The mutant AmiC protein was isolated from *P. aeruginosa* strain PAC452 harbouring the plasmid pSW1181. Protein was overexpressed and purified essentially as described (Wilson et al., 1991), but with 5 mM butyramide (Sigma) present in the culture medium and maintained in all buffers during purification. The mutant PAC181 AmiC protein is much less easily handled than the wild type, and the omission of butyramide from the growth medium and isolation buffers significantly decreased the solubility of the mutant AmiC and prevented effective purification.

Crystallization of PAC181-AmiC–butyramide complex

The solubility profile of the PAC181-AmiC protein in the presence of butyramide proved to be significantly different from that for the PAC1-AmiC with acetamide (Wilson et al., 1991), and a new crystallization condition was identified using a sparse matrix screen (Jancarik and Kim, 1991). Crystals of diffraction quality were eventually obtained from micro-batch experiments containing 12.8 mg/ml PAC181-AmiC, 5 mM butyramide, 1.36 M sodium citrate and 100 mM HEPES-NaOH (pH 7.5), implemented under paraffin oil in Terasaki dishes (Chayen et al., 1992).

X-Ray data collection, processing and refinement

Diffraction data to 2.7 Å resolution were collected at 100 K from a single PAC181-AmiC–butyramide co-crystal, cryoprotected by soaking in 1.5 M sodium citrate, 100 mM HEPES-NaOH and 25% (v/v) glycerol, on a 30 cm MAR Image Plate detector mounted on a Rigaku RU200 rotating anode X-ray generator. Diffraction images were integrated using the MOSFLM package (Leslie, 1995) and reduced using the SCALA, AGROVATA and TRUNCATE programs of the CCP4 Suite (CCP4, 1994). The scaled and merged data consist of 10 102 unique reflections, 97.8% complete in the range 38 to 2.7 Å (97.2% in the outer shell), with an average of 2.7 observations per reflection, and a merging R-factor of 0.098 (0.185 in the outer shell). Although the crystallization conditions for PAC181-AmiC with butyramide differ significantly from those for PAC1-AmiC with acetamide, the space group of the crystals is the same (P4 2212), and unit cell parameters (a, 104.15 Å; c, 65.68 Å) are very similar. Initial models were obtained by simulated annealing refinement of the coordinates (PDB code: 1PEA) for the PAC1-AmiC–acetamide complex (Pearl et al., 1994) against the PAC181-AmiC–butyramide complex data, using X-PLOR (Brünger, 1992), but with coordinates for the bound acetamide and solvent molecules omitted from the start model, and residue 106 modelled as alanine. Difference electron density maps were calculated with σA-weighted coefficients to minimize model bias (Read, 1986), and examined using ‘O’ (Jones et al., 1991). Subsequent simulated annealing and conjugate gradient refinement produced the current model consisting of 370 residues, 57 solvent molecules and a butyramide ligand. The crystallographic R-factor is 0.24 and the free-R factor with 5% of the data omitted from refinement is 0.30. The geometric parameters of the model are within the ranges expected at this resolution (Lasowski et al., 1993). Coordinates have been deposited in the Protein Data Bank with accession code 1QNL.

CD spectroscopy

Near-UV circular dichroism signals were measured on a nitrogen flushed Jasco J720 spectropolarimeter. Measurements were made with protein concentrations of 0.4 mg/ml and ligand concentrations of 10 mM using a 1 cm pathlength cuvette. The CD signal at 289 nm was measured as a function of temperature and fit to a van’t Hoff equation (Freeman et al., 1998). The PAC1-AmiC–acetamide complex displayed a Tm of 84°C; the PAC1-AmiC–butyramide complex a Tm of 79°C and the PAC181-AmiC–butyramide complex a Tm of 59°C.

Results

Characterization of PAC181 amidase regulatory genes

Amidase activities were determined for *P. aeruginosa* strains PAC1 (wild-type), PAC181 (butyramide inducible), PAC327

Table 1: Amidase activity of *P. aeruginosa* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Plasmid</th>
<th>Succinate</th>
<th>Succinate + butyramide</th>
<th>Succinate + lactamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC1</td>
<td>Inducible Ami⁺</td>
<td>—</td>
<td>0.1</td>
<td>8.1</td>
<td>0.2</td>
</tr>
<tr>
<td>PAC181</td>
<td>Butyramide inducible</td>
<td>—</td>
<td>0.1</td>
<td>0.8</td>
<td>1.58</td>
</tr>
<tr>
<td>PAC327</td>
<td>amiCR⁻</td>
<td>pSW181</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>PAC327</td>
<td>amiCR⁻</td>
<td>pSW181</td>
<td>0.1</td>
<td>9.7</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Activities are in μM acetylated amylate formed per min in a standard amidase assay (Drew, 1984)
Altered specificity of amide receptor AmiC

Fig. 2. Electron density for the 106Thr→Asn mutation. Electron density from an $F_o-F_c$ map with structure factors calculated from the wild-type PAC1-AmiC (PDB code: 1PEA) refined against the PAC181-AmiC diffraction data, with the ligand omitted, and only a $C_{\alpha}$ included for the side chain of residue 106. The resultant density (contoured at 1.25 Å) confirms the replacement of Thr by Asn at 106, as shown by sequencing of the PAC181-amiC gene, and the presence of butyramide.

Fig. 3. Detailed interactions in the AmiC binding site. (a) Acetamide bound to PAC1-AmiC. (b) Butyramide bound to PAC181-AmiC. The loss of the $C_{\alpha}$ of threonine, which delimits the side of the ligand-binding pocket in PAC1-AmiC, provides a space for the extra two carbons of the butyramide aliphatic chain. Hydrogen bonds are shown as broken cylinders.

(amiCR) and for PAC327 harbouring the plasmid pSW1181, which contains the 1.8 kb amiCR fragment amplified from PAC181 (see Materials and methods). PAC1 displayed characteristic lactamide inducibility, and PAC181 displayed lactamide and butyramide inducibility as previously described (Turberville and Clarke, 1981), but with lactamide inducibility at a lower level than in PAC1 (Table I). Despite the regulatory negative phenotype, there is some residual background amidase activity detectable in PAC327, but no induction is observed in the presence of lactamide or butyramide. PAC327 harbouring pSW1181 shows substantial induction of amidase activity in the presence of lactamide or butyramide, confirming that the butyramide inducible phenotype was retained in the cloned PAC181 amiCR fragment. Nucleotide sequencing of the entire fragment revealed only a single mutation from the wild-type PAC1 sequence, a C→A transversion at position 317 in the amiC gene.

Molecular basis of butyramide inducibility of PAC181-AmiC

Earlier studies of the amidase operon had demonstrated that the responsiveness to amide inducers was a function of the AmiC protein (Wilson et al., 1993). Phenotypic changes in the response to specific amides by mutant strains would therefore be expected to be accompanied by mutations in the AmiC protein rather than in other components of the operon. The nucleotide sequence of the amiC gene from the butyramide-inducible strain P. aeruginosa PAC181 revealed a single base change relative to the wild-type amiC gene sequence, resulting in the replacement of a threonine residue at 106 in the PAC1-AmiC sequence (SwissProt entry AMIC_PSEAE) with an asparagine. As residue 106 is located close to the amide binding site at the interface of the N- and C-terminal domains of AmiC (Pearl et al., 1994), a mutation in this residue would be consistent with a change in amide ligand specificity (Figure 1).

To define the structural effect of the Thr106→Asn mutation...
in PAC181, the crystal structure of PAC181-AmiC was determined in complex with butyramide. Difference Fourier maps calculated after refinement with a model in which only a Cα side chain was included for residue 106, clearly show electron density for asparagine rather than threonine (Figure 2), confirming the nucleotide sequence. Clear positive difference electron density is also present for a bound butyramide molecule whose amide head group and α-carbon are in essentially identical positions to the equivalent atoms of acetoamide bound to PAC1-AmiC.

The mutation of Thr106→Asn removes the γ-methyl group of threonine, which forms part of the side wall of the amide binding pocket in PAC1-AmiC, and thereby increases the volume of the amide-binding site. Butyramide binds to PAC181-AmiC in an unfavourable folded-over conformation in which the α and β methylene protons are eclipsed, and the γ-methyl group is in van der Waal’s contact with the plane of the amide head group. The β methylene group is in van der Waal’s contact with the γ-methyl of Thr 233, and the γ-methyl packs against the planes of the side chains of Asn106 and Tyr83 (Figure 3).

Adaptation to binding butyramide destabilizes AmiC

In the wild-type PAC1-AmiC, the side-chain γ-hydroxy group of Thr106 is hydrogen bonded to the peptide oxygen of Cys82 and weakly (3.2 Å), to the side-chain amide of Gln27. The γ-methyl of Thr106 is directed towards the amide-binding pocket, but does not make any direct contact with the bound acetoamide in that complex. In the mutant PAC181-AmiC, the side chain of the Asn106 is orientated so that its Cα-Cγ bond bisects the direction of the γ-hydroxyl and γ-methyl of the Thr106 in the PAC1-AmiC. The amide nitrogen in the head group of Asn106 picks up the hydrogen bond to the peptide oxygen of Cys82, and makes a strong hydrogen bond (2.7 Å) to the amide head group of Gln27, which changes conformation to make this interaction. Thus, the substitution of Asn for Thr at 106 retains the hydrogen binding interactions made by Thr106 in the wild-type PAC1-AmiC. However, the amide oxygen of Asn106 in the PAC181 mutant is left buried in a hydrophobic environment, packed against the side chain of Tyr83, and with no hydrogen bonds to the rest of the protein, or to any solvent molecules. The burial of this polar group without any compensating interactions would be expected to be highly unfavourable. Consistent with this, the melting temperature (Tm) measured by near-UV CD (see Materials and methods) for denaturation of PAC181-AmiC with butyramide bound is down-shifted 20 K compared with the Tm of the PAC1-AmiC with butyramide, which is a poor ligand for the wild-type AmiC. Compared with the Tm for PAC1-AmiC with bound acetoamide, an optimal ligand, the Tm for PAC181-AmiC with butyramide is decreased by 25 K (Figure 4).

Discussion

Before the advent of recombinant DNA technology, biological selection and screening of mutagenized cultures was the only way in which molecular evolution could be studied. With the *P. aeruginosa* amidase, an advantage at the outset was the differing inducer and substrate specificities of the system (Kelly and Clarke, 1962). A large number of both regulatory and structural gene mutations were isolated which allowed growth on novel amide substrates (Clarke and Drew, 1988). These novel abilities have been attributed to changes in the amidase itself and to those in the negative regulator, ligand receptor, AmiC. AmiC, a member of the SMBP family, contains a widely used and adaptable ligand binding site. It appears that a single amino acid substitution in a residue close to the binding site in the AmiC of the butyramide inducible strain PAC181 is solely responsible for the new phenotype. PAC181 also contains a single residue substitution within the amidase enzyme itself; Ser→Phe at position 7. This gives an enzyme with higher activity towards butyramide hydrolysis (Turberville and Clarke, 1981), although the structural basis for this remains to be determined. PAC181 thus represents a new strain able to use butyramide, a repressor of the wild-type system, to induce expression of a new butyramide hydrolysing enzyme.

In adapting to butyramide induction via the Thr106→Asn mutation, PAC181-AmiC undergoes a significant decrease in its thermostability compared with wild type, and its half-life in *vivo* would be expected to be correspondingly shorter. However, the selective pressure to which the PAC181-AmiC sequence is a response, requires only that the amidase operon be inducible by butyramide. Thus, so long as the mutant AmiC can be synthesized at a sufficient level, fold and fulfil its biological role, its absolute structural stability need not be optimal. It is protein function that is selected in evolution, not structural stability, a fact often neglected in theoretical analyses of protein structure.

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References


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