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Antitermination of amidase expression in *Pseudomonas aeruginosa* is controlled by a novel cytoplasmic amide-binding protein

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Amide-inducible expression of the aliphatic amidase system of *Pseudomonas aeruginosa* can be reconstituted in *Escherichia coli* with only the amidase structural gene *amiE*, the negative regulator *amiC* and the positive regulator *amiR*, a transcription antitermination factor. Complementation experiments in *E.coli* suggest that negative control of amidase expression by AmiC is mediated by a protein-protein interaction with AmiR. Purified AmiC binds acetamide with a K_D of 3.7 μM in equilibrium dialysis studies, and therefore AmiC appears to be the sensory partner of the AmiC/AmiR pair of regulatory proteins, responding to the presence of amides. Sequence analysis techniques suggest that AmiC is a member of the structural family of periplasmic binding proteins, but has a distinct and novel cytoplasmic role.

Key words: binding proteins/gene regulation/signal transduction

Introduction

Pseudomonas aeruginosa is able to grow on short chain aliphatic amides by virtue of a chromosomally located amidase (EC 3.5.1.4) (Brammar and Clarke, 1964). Amidase activity is inducible by some low molecular weight amides, although the substrate and inducer specificities are quite distinct (Kelly and Clarke, 1962). The amidase genes have been cloned from a constitutive mutant PAC433 (Drew *et al.*, 1980) and from the wild-type strain PAC1 (Wilson and Drew, 1991). The *amiE* gene encoding the amidase enzyme was initially located (Clarke *et al.*, 1981) and sequenced (Brammar *et al.*, 1987) and two regulatory genes, *amiC* and *amiR*, which lie ~2 kb downstream from the *amiE* gene, have also been identified and sequenced (Cousens *et al.*, 1987; Lowe *et al.*, 1989; Wilson and Drew, 1991) (Figure 1).

The *amiR* gene was initially identified as a positive regulator of amidase expression (Farin and Clarke, 1978) and subsequently shown to encode a transcription antitermination factor, thought to function by allowing RNA polymerase to read through a rho-independent terminator identified between the *amiE* promoter and the *amiE* structural gene itself (Drew and Lowe, 1989). The second regulatory protein, AmiC, negatively regulates amidase expression and disruption of the *amiC* open reading frame leads to constitutive amidase expression. The *amiC* and *amiR* open reading frames overlap by 2 bp and are transcribed on the same mRNA (Wilson, 1991; Wilson and Drew, 1991). The AmiC protein has been overexpressed, purified and crystallized (Wilson *et al.*, 1991).

Previous complementation studies have shown that antitermination of *amiE* transcription by AmiR is independent of inducing amides (Cousens *et al.*, 1987; Wilson and Drew, 1991). This suggested that AmiC was the sensory protein and would be responsive to the presence of amides. The precise molecular mechanism of negative control by AmiC has not yet been fully elucidated.

Two well characterized bacterial regulatory systems, the *bgl* operon of *Escherichia coli* and the sac operon of *Bacillus subtilis*, also operate by transcription antitermination (Schnetz and Rak, 1988; Le Coq *et al.*, 1989). In the *bgl* operon the transcription antitermination factor BglG binds to a sequence in the nascent mRNA upstream of and overlapping with the transcription terminator and allows RNA polymerase to read through the terminator (Houman *et al.*, 1990). In both systems the antiterminator activity is negatively regulated by a membrane bound protein (BglF and SacX respectively) and these two proteins each form a part of a phosphoenolpyruvate dependent phosphotransferase system (Amster-Choder *et al.*, 1989; Le Coq *et al.*, 1989). The antitermination factors and negative regulators of both these systems show significant sequence homology. Under non-inducing conditions, the negative regulator BglF phosphorylates BglG, inhibiting its antitermination activity and under inducing conditions, BglF dephosphorylates BglG, activating it and also phosphorylating incoming β-glucosides.

The *P.aeruginosa* amidase operon is clearly distinct from the *bgl* and *sac* operons since the negative regulator AmiC is a soluble cytoplasmic protein, and neither AmiC nor AmiR show homology with their functional counterparts in the *bgl* and *sac* systems.

In this paper we show that a fully functional *P.aeruginosa* amidase induction system can be reconstructed using two and three-plasmid systems in *E.coli*, from the *amiE*, *amiC* and *amiR* genes alone. We also show that the AmiC protein is a specific amide binding protein and that its amide dependent inhibitory action on AmiR operates via a post-transcriptional interaction. We have identified AmiC as a member of the structural protein family comprising the bacterial periplasmic binding proteins, using sequence

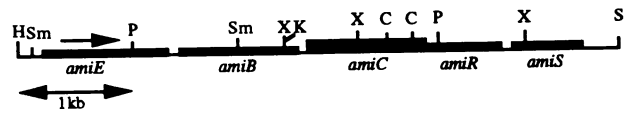

Fig. 1. Organization of the *P.aeruginosa* amidase operon. Restriction sites are indicated as follows: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; Sm, *Sma*I; X, *Xho*I. The *amiB* and *amiS* genes are uninvolved in regulation of amidase expression, but may form an active transport system for amides (Drew and Wilson, 1992).

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analysis techniques. The cytoplasmic location of AmiC suggests a so far unique role for a member of this protein family.

Results

Reconstitution of an inducible amidase expression system in E.coli

To establish that all of the regulatory genes for inducible amidase expression had been identified, we have reconstructed the regulatory system in *E.coli* (which lacks an aliphatic amidase activity) using a two-vector system. Plasmid pMW21 which expresses the *amiC* and *amiR* genes at high levels, was constructed by insertion of a *KpnI–SalI* fragment of the amidase operon into the broad host range expression vector pMMB66HE (Figure 2). This vector allows expression of *amiC* and *amiR* from the *tac* promoter under the control of IPTG. A second reporter plasmid (pTM1) was constructed which carries the *amiE* gene in pACYC184 under control of its native promoter and transcription terminator (Figure 2). Amidase activity was measured in *E.coli* JA221 harbouring these plasmids, after growth under inducing, non-inducing and repressing conditions (Table I). pTM1 (containing only the *amiE* gene and regulatory elements) expresses a low level of amidase activity which shows no significant increase in the presence of inducing (lactamide) or repressing (butyramide) amides. *Escherichia coli* simultaneously harbouring pTM1 and pMW21 (containing the *amiC* and *amiR* genes) shows a low level of amide-inducible amidase activity in the absence of IPTG presumably due to leaky repression of the *tac* promoter, and high amide-inducible levels of amidase expression in the presence of IPTG. Thus we have reconstituted inducible amidase expression in *E.coli* using the *amiE*, *amiC* and *amiR* genes alone. Inducible amidase expression can also be reconstructed in *P.aeruginosa* using pMW21 in strain PAC327 (Brown, 1969). PAC327 is *amiRC*$^-$ and amidase negative. In the presence of IPTG, PAC327 harbouring pMW21 shows inducible amidase expression (Table I).

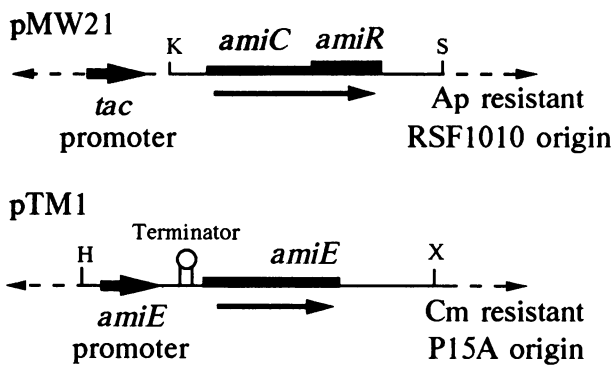

Fig. 2. Two-plasmid system. Structure of *amiC+amiR* expression plasmid pMW21, and *amiE* reporter plasmid pTM1. Restriction sites are as Figure 1. Selective antibiotics: Ap, ampicillin; Cm, chloramphenicol. *P.aeruginosa* derived sequences are shown as full lines, plasmid sequence as broken arrowed lines. The thin arrows indicate the direction of transcription.

Three-plasmid complementation in E.coli

Several possibilities exist for the mechanism of negative control of amidase expression by *amiC*. First, AmiC might be a conventional DNA binding repressor of *amiE* or *amiR* transcription; however, the AmiC sequence contains none of the motifs usually associated with sequence specific DNA binding proteins, nor does purified AmiC bind to DNA (Wilson, 1991). Alternatively, AmiC may interact directly with AmiR and modify its activity either by formation of a stoichiometric complex, or by an enzymatic modification of AmiR such as phosphorylation, as seen in the *bgl* and *sac* operons (s.v.).

To distinguish between these two modes of AmiC action, a three-plasmid complementation system was constructed in *E.coli* with *amiE*, *amiC* and *amiR* each carried on separate compatible plasmids. The *amiE* gene was carried on pACYC184 (pTM1) described above. The *amiC* gene (*KpnI–PvuII*) was carried on pMMB66HE (pSW41) and expressed from the vector *tac* promoter (Wilson and Drew, 1991) and *amiR* (*XhoI–XhoI*) was carried on pBGS19$^-$ (pTM2) with *amiR* expressed from the vector *lac* promoter (Figure 3).

Amidase activities expressed by *E.coli* with pTM1 (*amiE* alone), pTM1+pTM2 (*amiE+amiR*) and pTM1, pTM2 and pSW41 (*amiE+amiR+amiC*) are shown in Table II. As in the two-plasmid experiments, pTM1 alone expressed low levels of amidase activity. With the addition of plasmid pTM2, high levels of amidase activity were seen both in the presence and absence of amides. With the addition of the third plasmid pSW41, which produces very high constitutive levels of AmiC, there is almost complete shut down of amidase expression. Since AmiR and AmiC are transcribed from vector promoters, it is most unlikely that AmiC can be affecting the expression of the *amiR* gene by repression of its transcription, but is rather acting post-transcriptionally by interaction with the AmiR protein itself.

Table I. Amidase activity in *E.coli* and *P.aeruginosa* carrying recombinant plasmids

Strains/ plasmids	IPTG	Glucose	Glucose lactamide	Succinate	Succinate/ lactamide	Succinate/ butyramide
E.coli[a]						
pTM1	–	0.5	0.5	0.4	0.7	1.3
pTM1, pMW21	–	0.8	4.9	0.8	6.4	0.3
pTM1, pMW21	+	3.0	22.3	3.9	40.4	0.5
P.aeruginosa[b]						
–	–	–	–	0.4	0.3	–
pMW21	+	–	–	0.7	3.0	–

[a]*E.coli* strain JA221.
[b]*P.aeruginosa* strain PAC327 (*amiCR*$^-$).

In this three-plasmid complementation system, negative regulation by AmiC cannot be relieved by addition of inducing amides. Although expression of *amiR* from pTM2 produces a significant *in vivo* effect on amidase expression, it does not produce high levels of AmiR, and no new band is visible on SDS−PAGE gels (Wilson, 1991), whereas the AmiC expression vector pSW41 produces a major new band on SDS−PAGE gels. It is likely that the unrelievable inhibition of amidase expression in the three-plasmid system is due to the presence in the cell of saturating amounts of AmiC with respect to AmiR.

Amide binding by AmiC

Complementation systems containing *amiE* and *amiR* alone do not respond to amide inducers, whereas the addition of *amiC* confers amide inducibility, implicating the AmiC protein as the amide 'sensor', and suggesting that AmiC should bind amides. Equilibrium dialysis experiments were performed using purified AmiC (Wilson *et al.*, 1991) and [^{14}C]acetamide. A Scatchard plot (Figure 4) of the results from these experiments gives a value for K_D of 3.7 μM and a protein:ligand stoichiometry of 2:1. As the AmiC protein has been found to migrate as a dimer in gel filtration studies (Wilson *et al.*, 1991) this ratio suggests that one acetamide molecule binds to an AmiC dimer. Other amides, lactamide and propionamide which have been found to be strong inducers of amidase expression compete with acetamide for binding to AmiC, as does butyramide, an inhibitor of induction (Kelly and Clarke, 1962). In competition binding experiments, propionamide shows a K_D of 3.1 μM whereas lactamide and butyramide bind ~100-fold less tightly.

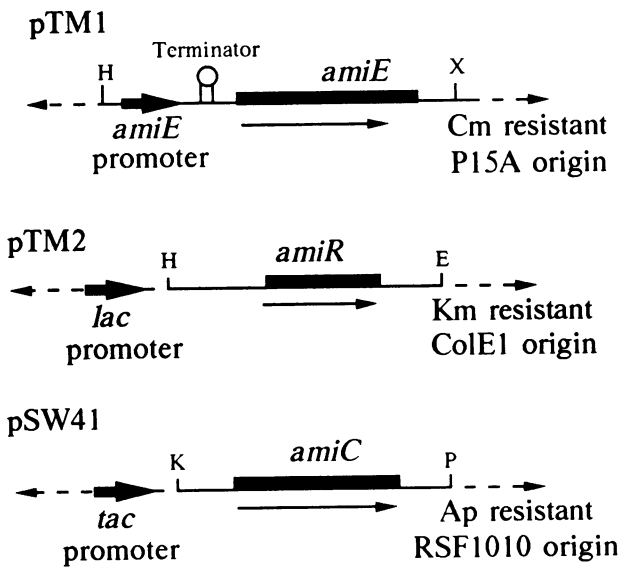

Fig. 3. Three-plasmid system. Structure of *amiE* reporter plasmid pTM1 (see Figure 2), *amiR* expression plasmid pTM2 and *amiC* expression plasmid pSW41. Restriction sites are indicated as in Figure 1; selective antibiotics as Figure 2 plus Km, kanamycin.

Sequence analysis of the AmiC protein

The derived amino acid sequence of the AmiC protein (Wilson and Drew, 1991) was compared with 26 706 sequences in the SwissProt sequence database, using the FASTA program (Pearson and Lipman, 1988). A relatively weak match (19.2% identity over 339 amino acids) was found with the sequence of the BraC branched-chain amino acid binding protein of *P.aeruginosa* (Hoshino and Kose, 1989). Figure 5 shows an alignment of the AmiC sequence with the BraC protein and several functionally related proteins. Although the individual identity between AmiC and the branched-chain amino acid binding proteins is low, AmiC shows a general overall similarity to the family of proteins over its entire length, with conformationally important residues such as glycine and proline, being frequently conserved. The similarity of AmiC to these proteins has been further tested by secondary structure prediction. A consensus of seven standard prediction algorithms (Eliopoulos *et al.*, 1982) was used to predict the secondary structure of AmiC and of the leucine-isoleucine-valine binding protein of *E.coli* (LivJ). These predictions were compared with the secondary structure actually observed in the crystal structure of LivJ (Sacks *et al.*, 1989) (Figure 4). While the predicted secondary structure of LivJ does not precisely match the observed structure, most secondary structural elements are correctly predicted in position and type, if not in length. Most interestingly, the predicted secondary structure of AmiC corresponds equally well to the observed LivJ secondary structure. As a final clue to the structure of AmiC, we have submitted the amino acid sequence to a 'threading' analysis (Jones *et al.*, 1992). This procedure measures the 'fit' of the AmiC sequence onto 102 three-dimensional folds from known X-ray structures, using a combination of solvent accessibility and pair-distance parameters. The distribution of threading scores for AmiC is shown in Figure 6. The match with most folds gives a roughly Gaussian distribution; however, the scores for two related folds, the *E.coli* LivJ protein (Sacks *et al.*, 1989) and the *Salmonella typhimurium* galactose binding protein (Mowbray and Petsko, 1983), are significantly better and outlie the overall distribution. Taken together these analyses suggest that the AmiC sequence is capable of adopting a three-dimensional structure which is very similar to LivJ and the *S.typhimurium* galactose binding protein, both members of a large and well characterized structural family of binding proteins (Quicho, 1991) which function as periplasmic receptors for small molecules in many bacteria.

Discussion

Reconstruction of the *P.aeruginosa* amidase regulatory system in *E.coli* confirms that only two regulatory proteins

Table II. Amidase activity in *E.coli* JA221 carrying *amiE*, *amiC* and *amiR* recombinant plasmids

Plasmids	Glucose	Glucose lactamide	Succinate	Succinate/ lactamide	Succinate/ butyramide
pTM1	0.5	0.5	0.4	0.7	1.3
pTM1, pTM2	30.8	31.8	31.0	33.6	39.1
pTM1, pTM2, pSW41	0.9	1.0	0.9	0.9	0.9

are required for amide-inducible expression, and identifies the AmiC protein as the component responsible for recognition of inducer molecules. This role for AmiC has been confirmed by demonstrating binding of acetamide and other inducers/induction inhibitors by purified AmiC *in vitro*. In the three-plasmid complementation system AmiC is able to block AmiR activity even though AmiR was expressed from a foreign promoter. We conclude therefore that AmiC does not function as a classical DNA binding repressor, but inhibits the action of AmiR post-transcriptionally (Wilson and Drew, 1991). Analysis of the AmiC sequence shows it to be a member of a structural family usually found as periplasmic receptors in a variety of bacterial transport and chemotaxis systems. AmiC lacks the periplasmic localization signal found at the N-terminus of these proteins, consistent with its cytoplasmic localization. The classic periplasmic binding proteins display a characteristic ligand dependent protein–protein interaction with a specific membrane bound protein complex (Furlong, 1987). As AmiC has a structural homology and displays a ligand binding function of similar affinity to these proteins, it too might act via a protein–protein interaction but with the cytoplasmic antitermination factor AmiR, and this interaction may be responsible for inhibition of the antitermination activity of AmiR.

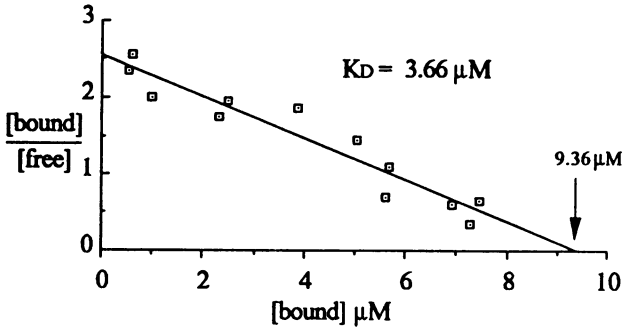

Fig. 4. Scatchard plot of [^{14}C]acetamide binding to AmiC. See text for experimental details. The binding constant was determined by linear regression of the experimental points. The arrow indicates the theoretical maximum acetamide binding.

We have attempted to determine whether AmiC covalently modifies AmiR by phosphorylation or by proteolysis, but no such activity has been detected (data not shown), suggesting that AmiC inhibits AmiR by direct binding,

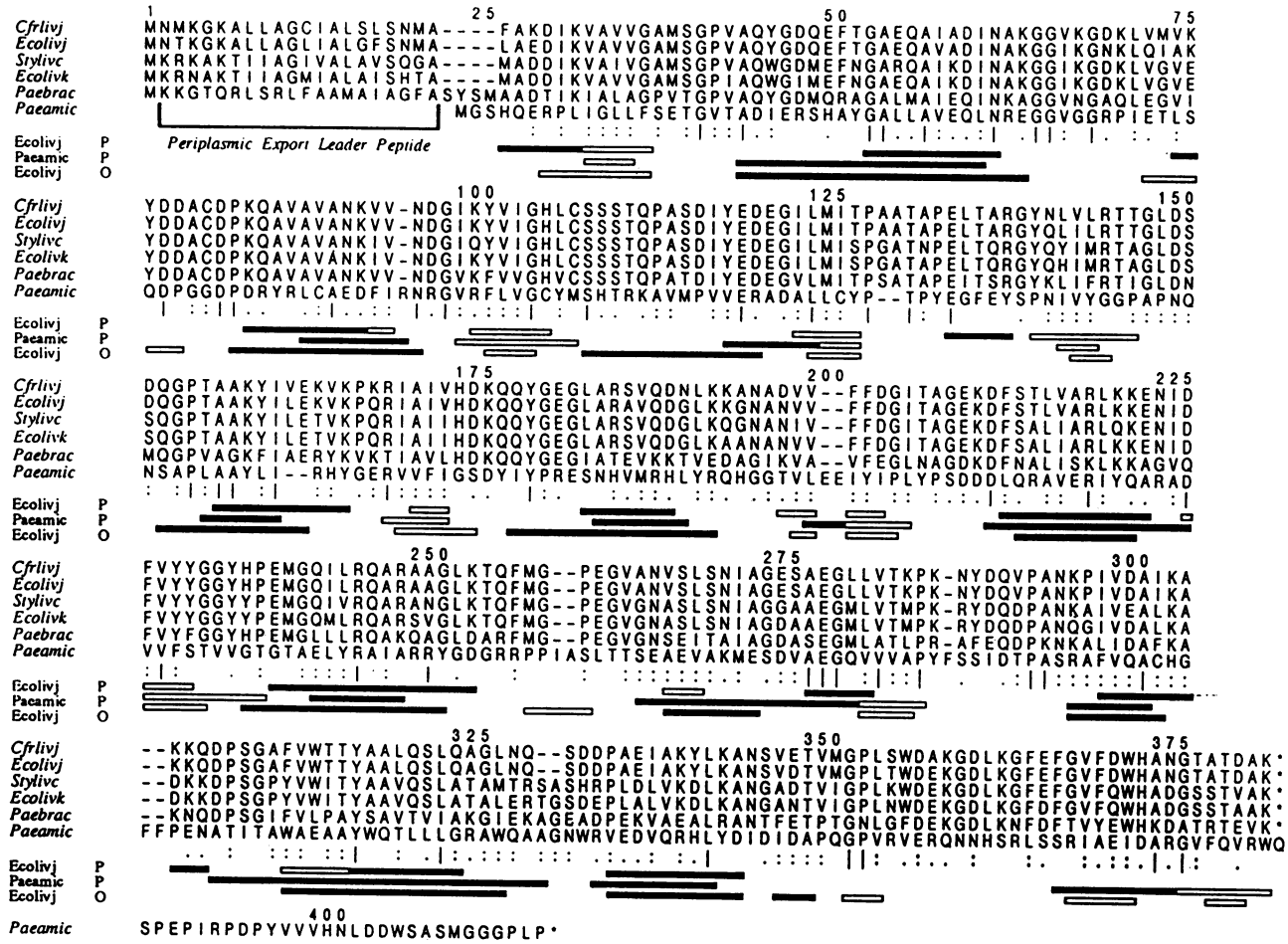

Fig. 5. Sequence alignment of AmiC with members of the periplasmic binding protein structural family. Sequences: Cfrlivj, *C.freundi* LivJ (Garvin and Hardies, 1991); Ecolivj, *E.coli* LivJ (Sacks *et al.*, 1989). Stylivc, *S.typhimurium* LivC (Ohnishi *et al.*, 1990); Ecolivk, *E.coli* LivK (Adams *et al.*, 1990); Paebrac, *P.aeruginosa* BraC (Hoshino and Kose, 1989); Paeamic, *P.aeruginosa* AmiC (Wilson and Drew, 1991). Sequence homologies are indicated below the sequences by |, totally conserved; :, strongly conserved; ., weakly conserved. Secondary structure predictions/observations are Ecolivj P, *E.coli* LivJ predicted secondary structure; Paeamic P, *P.aeruginosa* AmiC predicted secondary structure; Ecolivj O, *E.coli* LivJ observed secondary structure. Solid bars, α-helix; open bars, β-sheet. The N-terminal periplasmic export signal found in the periplasmic binding protein sequences is indicated.

blocking access of AmiR to the *amiE* RNA leader sequence. Previous genetic studies suggest that there is a stoichiometry between the levels of AmiC and AmiR. Overexpression of AmiR in a wild-type *P.aeruginosa* background leads to constitutive amidase expression (Wilson and Drew, 1991). In this situation there is only a single copy of *amiC* on the chromosome expressing relatively low levels of AmiC compared with the overexpressed plasmid-borne *amiR* gene. Similarly, if AmiC is overexpressed with respect to AmiR in *P.aeruginosa*, the system becomes uninducible and all amidase expression ceases. Coordinate overexpression of AmiC and AmiR in *E.coli* and in *P.aeruginosa* gives normal inducible amidase expression. Taken together these results suggest that the mechanism of AmiC inhibition of AmiR operates via a stoichiometric rather than a catalytic relationship between the two proteins, probably involving formation of an AmiC–AmiR complex.

On binding their specific ligand, some classical periplasmic binding proteins display a conformational change which enables them to bind to the membrane protein complex, and which can be detected spectroscopically (e.g. Zukin, 1979). We have attempted to observe a similar change in the conformation of AmiC on binding of acetamide, but no significant signal has been observed using fluorescence or near-UV circular dichroism (data not shown), however, both these techniques are only sensitive to relatively large changes in the environments of aromatic groups, and the lack of a significant signal does not indicate the absence of some more subtle structural change on binding of amides. Addition of amides does not alter the dimerization state of AmiC, which runs as a dimer of molecular weight 86 kDa, even in the presence of >100 mM acetamide. As AmiC is the receptor of amides in the amidase system, and its ability to inhibit the action of AmiR depends on the presence or absence of amides, it seems likely that binding of acetamide to AmiC will have some effect on its structure, if only in that part that interacts with AmiR.

A full understanding of the molecular mechanism of this unusual signal transduction/gene regulation system must await the successful purification of AmiR for studies of the AmiC–AmiR complex in the presence of inducing/induction inhibiting amides.

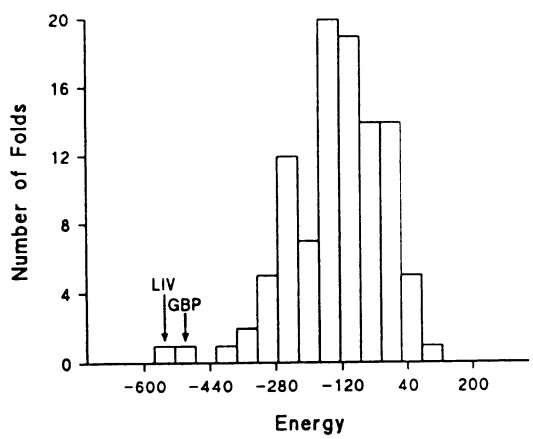

Fig. 6. Threading analysis of the AmiC sequence. Histogram of pseudo-energies for the AmiC sequence threaded on to 102 known protein folds. The most stable threadings on to the folds of *E.coli* LivJ and *S.typhimurium* galactose binding protein are indicated. The score for the LivJ threading is >100 kcal/mol more stable than that for the galactose binding protein and suggests a strong similarity between the AmiC and LivJ three-dimensional structures.

Materials and methods

Construction of plasmids

All plasmid purifications, transformations and cloning was carried out as described previously (Wilson and Drew, 1991). Plasmid pTM1 was constructed by insertion of a 2.3 kb *Hin*dIII–*Xho*I *amiE* fragment from pAS20 (Wilson and Drew, 1991) into the *Hin*dIII and *Sal*I sites of pACYC184 which has a p15A origin of replication and confers chloramphenicol resistance. Plasmid pTM2 carries a 1.5 kb *Hin*dIII–*Eco*RI fragment from pSW24 (Wilson, 1991) subcloned into pBGS19$^-$, which has a ColE1 origin of replication and confers resistance to kanamycin. The orientation of the *amiR* gene allows expression from the *lac* promoter in the plasmid.

Assay of amidase activity

Amidase activity in intact cells was measured by the transferase assay (Brammar and Clarke, 1964) with acetamide as the substrate. Activity levels presented in this article are the mean values of duplicate assays carried out on at least three separate occasions. One unit represents 1 μmol of acetohydroxamate formed per min per mg of bacteria.

Purification of AmiC

AmiC was purified as described previously (Wilson *et al.*, 1991) and protein concentration determined by the Bradford assay (Bradford, 1976).

Equilibrium dialysis with acetamide

[^{14}C]acetamide was synthesized by reaction of [^{14}C]ethyl acetate (Amersham International) with an excess of concentrated ammonia in ethanol and cold ethyl acetate. The product from an identical cold synthesis was characterized by assay with *P.aeruginosa* amidase enzyme and found to be >99.5% pure. In all equilibrium dialysis experiments a constant concentration of AmiC was used (18 μM) and the concentration of [^{14}C]acetamide was varied. In competition dialysis experiments, constant concentrations of AmiC (18 μM) and [^{14}C]acetamide (15 μM) were used, and the concentrations of competing amides were varied. To determine accurately the concentration of the radioactive acetamide, a titration experiment was performed in which non-radioactively labelled acetamide of known concentration was used as competitor of AmiC binding. The concentration of cold acetamide which gave a 50% reduction in the amount of bound acetamide was taken as an equimolar concentration to the labelled acetamide (data not shown). Dialysis experiments were performed with 50 μl volumes on either side of a 14 kDa cut-off dialysis membrane in a Teflon equilibrium dialysis module (Hoeffer Scientific) at 16°C overnight. Duplicate 20 μl samples were taken from both sides of the membrane and liquid scintillation counted.

Sequence alignment, secondary structure prediction and fold analysis

Sequence alignments were performed using the multiple alignment algorithm of Feng and Doolittle (1987) implemented in the program PileUp (University of Wisconsin) with small manual adjustment of the final alignments. Secondary structure prediction was performed using the consensus prediction method of Eliopoulos *et al.* (1982). Optimal fold threading of the AmiC sequence used the method of Jones *et al.* (1992). Briefly, the AmiC sequence was threaded on to 102 protein folds, and the pseudo-energy of each alignment calculated as the weighted sum of pairwise and solvation pseudo-energy terms.

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