Crystal structure of a D-aminopeptidase from Ochrobactrum anthropi, a new member of the ‘penicillin-recognizing enzyme’ family

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Background: β-Lactam compounds are the most widely used antibiotics. They inactivate bacterial DD-transpeptidases, also called penicillin-binding proteins (PBPs), involved in cell-wall biosynthesis. The most common bacterial resistance mechanism against β-lactam compounds is the synthesis of β-lactamases that hydrolyse β-lactam rings. These enzymes are believed to have evolved from cell-wall DD-peptidases. Understanding the biochemical and mechanistic features of the β-lactam targets is crucial because of the increasing number of resistant bacteria. DAP is a D-aminopeptidase produced by Ochrobactrum anthropi. It is inhibited by various β-lactam compounds and shares ~25% sequence identity with the R61 DD-carboxypeptidase and the class C β-lactamases.

Results: The crystal structure of DAP has been determined to 1.9 Å resolution using the multiple isomorphous replacement (MIR) method. The enzyme folds into three domains, A, B and C. Domain A, which contains conserved catalytic residues, has the classical fold of serine β-lactamases, whereas domains B and C are both antiparallel eight-stranded β barrels. A loop of domain C protrudes into the substrate-binding site of the enzyme.

Conclusions: Comparison of the biochemical properties and the structure of DAP with PBPs and serine β-lactamases shows that although the catalytic site of the enzyme is very similar to that of β-lactamases, its substrate and inhibitor specificity rests on residues of domain C. DAP is a new member of the family of penicillin-recognizing proteins (PRPs) and, at the present time, its enzymatic specificity is clearly unique.

Introduction

β-Lactam antibiotics kill bacteria by interfering specifically with the biosynthesis of peptidoglycans, the major constituents of the bacterial cell wall. This polymer is unique to bacteria and this explains the high specificity of β-lactam targets (Figure 1) and their relatively low toxicity towards eukaryotic cells. The physiological targets of β-lactam compounds are membrane-bound DD-peptidases (usually called penicillin-binding proteins or PBPs) that are responsible for the synthesis and remodelling of the peptidoglycan. These peptidases are active-site serine enzymes and perform their catalytic cycle according to an acylation/deacylation mechanism. β-Lactam antibiotics acylate the active-site serine residue of the DD-peptidases, forming rather stable covalent noncatalytic acyl enzymes [1].

One of the resistance mechanisms utilised by bacteria against these compounds is the production of β-lactamases that catalyse the irreversible hydrolysis of the amide bond of the β-lactam ring, thus yielding inactive products [2]. Most β-lactamases are active-site serine enzymes and follow a catalytic pathway similar to that of DD-peptidases. Despite very low degrees of sequence identity, striking structural similarities have been highlighted between serine β-lactamases and DD-peptidases by comparing several X-ray crystallographic structures of PBPs and class A and C β-lactamases [3–14]. The structures of these enzymes can be partly superimposed, most strikingly at the level of the active centres [4]. They all exhibit a single domain containing two regions, one α/β and one all-helical, with the catalytic site lying between them. In the immediate vicinity of the active-site serine residue of DD-peptidases and β-lactamases, three elements, conserved in all active-site serine penicillin-recognizing enzymes (PRPs, including the PBPs which covalently bind β-lactams), have been identified that appear to be directly or indirectly involved in the substrate recognition and catalytic processes [14]. The first element (Ser–X–X–Lys sequence, where X is any amino acid)
DAP can be considered a PRP on the basis of its primary structure and the fact that it is inhibited by β-lactam compounds. It has been isolated from strain SCRC C1–38 and displays strict d-stereospecificity towards low molecular weight d-amino acid amides, d-alanine β-alkylamides and peptides with a d-alanine at the N terminus [19,23,24]. DAP has a molecular weight of ~120,000 and is composed of two identical subunits (M_r = 54,000). The corresponding gene has been isolated from O. anthropi and its nucleotide sequence determined [19]. The deduced amino acid sequence of the enzyme shows that it shares 25% and 22% identity with the Streptomyces R61 dD-carboxypeptidase (R61) and the class C β-lactamase from Escherichia coli, respectively [23], including the three conserved elements described above. Site-directed mutagenesis experiments [23] show that residues Ser62 and Lys65 are essential for catalysis and that Cys61 is probably involved in the substrate-binding step.

The resolution of the structure of DAP will supply an additional structure in the family of PRPs and should be especially interesting when one considers the unique substrate profile of this enzyme. Furthermore, the enzymes specific for d-amino acids are poorly characterised, and the study of d-aminopeptidases is of interest for enzymatic stereoselective synthesis in organic chemistry. We present here the structure of the d-aminopeptidase from O. anthropi SRC C1–38, solved at 1.9 Å resolution, and its comparison with the structures of R61 [11,12], the class A β-lactamase from Bacillus licheniformis [13] and the class C β-lactamase from Enterobacter cloacae P99 [14]. The DAP structure reveals the presence of a ‘serine-β-lactamase’ catalytic domain and of two additional domains, one of which is likely to be responsible for the specific substrate recognition and interaction with β-lactam compounds.

**Results and discussion**

**Analysis of the structure**

DAP has a dimeric structure, each monomer having dimensions of ~67 × 60 × 47 Å (Figure 2). The monomer consists of three domains (Figures 2a,b). The N-terminal catalytic domain (domain A, residues 3–331) folds into two regions, one αβ and one helical region. The αβ region is formed by residues 3–62 and 182–331 and consists of a main five-stranded antiparallel β sheet flanked by three helices. Two additional antiparallel β strands (S3–S4) lie perpendicular to the main β sheet. The all-helical region (residues 63–181) contains eight helices (Figures 2a,b).

The structure of this domain is analogous to that of R61 and serine β-lactamases, which is consistent with the sequence alignment results [23] (Figure 3).

Two additional domains, B (residues 341–418) and C (residues 422–520), complete the structure. They are both constituted by eight antiparallel β strands organised in β barrels. Domain B is connected to domain A by an
Figure 2

Overall structure of DAP. Ribbon diagrams showing (a) the DAP dimer and (b) the DAP monomer. Domains A, B and C are coloured blue, green and orange, respectively. The γ and Ω loops are purple and red, respectively. (c) Stereoview of the Cα trace of the DAP monomer. The figures were produced using the program MOLSCRIPT [44], Raster3D [45] and TURBO–FRODO [42].

Figure 3

Comparison of the folds of (a) the catalytic domain of DAP, (b) the α-carboxypeptidase from Streptomyces sp. R61 [11], (c) the class A β-lactamase from B. licheniformis [13], and (d) the class C β-lactamase from E. cloacae P99 [14]. β Strands and α helices are blue and red, respectively. The Ω loop lies at the lower centre of each molecule. The figures were produced with the programs MOLSCRIPT [44] and Raster3D [45].
eight-residue peptide (residues 332–340). This domain forms five hydrogen bonds with domain A through loop 408–411, as well as three hydrogen bonds, hydrophobic contacts and two salt bridges with domain C through loop 406–410 and residue 372, respectively. Domain C is connected to domain B by a short link (residues 419–421). In contrast, domain C makes close contacts with the helical region of the catalytic domain. Helix H5 of the catalytic domain protrudes into domain C making extensive hydrophobic interactions with aromatic and aliphatic sidechains that cover the cavity of the β barrel. Similarly, the 476–486 loop of domain C, which will be named the ‘γ loop’ hereafter, lies between the helical and the α/β regions of domain A, thus forming part of the wall of the catalytic pocket (see below). Interactions between domains A and C are mostly hydrophobic but two salt bridges and 12 hydrogen bonds can also be identified.

Substrate/inhibitor recognition and binding
R61 and DAP catalyse the hydrolysis of a peptide bond between two D-amino acids situated respectively at the C- and N-terminal ends of a peptide substrate. They probably use similar catalytic mechanisms involving conserved residues [23]; however, they each specifically recognise and bind only one of the two charged ends of the peptide substrate. The environment of the active site is expected to provide positive charges in R61 and negative charges in DAP, in order to bind negatively and positively charged substrates, respectively.

The active site of R61 contains several basic groups [11] and is fairly open to the solvent. An analysis of the electrostatic potential surface in R61 reveals that the substrate-binding site of the enzyme is positively charged (Figure 4a). These charges are supposed to be responsible for the specific recognition of the C terminus of the peptide substrate by the enzyme and, in part, for its lack of aminopeptidase activity. The Arg285 sidechain, situated in the vicinity of the catalytic site, is probably responsible for the specific recognition of the substrate carboxylate (Figure 4d), as it is the only positively charged residue situated in a good position to do so. In the structure of DAP, this residue is replaced by Asn275 that is too far away from the catalytic centre to participate in substrate binding (Figure 4d).

Compared to R61 (and β-lactamases of classes A and C), the catalytic site of DAP is less accessible to the solvent owing to the presence of the C-terminal domains of the enzyme (Figure 4c). It is situated in the bottom of a crevice located at the interface between domain C and the helical and the α/β regions of domain A. In contrast to those of R61 or of β-lactamases (usually positively charged), the substrate-binding site of DAP contains only...
one charged residue (Figure 4c), Asp481, situated on the other side of the cleavage site compared to Arg285 in R61. It is well positioned to stabilise the positive charge of the substrate (Figures 4b,c). Asp481 is situated on the domain C γ loop and might be responsible for the specific recognition of the N terminus of the substrate.

Enzymological studies have shown that the affinity of DAP is higher for unsubstituted β-lactam compounds than for those in which the amino group is blocked by acyl moieties [23] (Figure 1). As discussed above, when compared with R61, the additional domains in the DAP structure considerably reduce the accessibility of the active site for potential ligands (Figures 4a,c). The structures of covalent complexes formed between R61 and two cephalosporins (cefalothin and cefotaxime) (Figure 1c) reveal the importance of three threonine residues (Thr116, Thr299 and Thr301) that are directly involved in the binding of these β-lactam antibiotics [12,15]. In DAP, these residues are replaced by Trp114, Gly288 and Ala290, respectively (Figure 5a), thereby reducing the number of possible interactions with these inhibitors. Moreover, the sidechains of N-substituted β-lactams, as in cefotaxime, would create short contacts with the γ loop. Accordingly, Asano et al. [23] have shown that DAP is inhibited by 7-ACA and 6-APA (Figures 1a,b) with $K_i$ values of 0.1 mM and 0.3 mM, respectively. The active-site topology of DAP indicates that such unsubstituted β-lactams can bind in the same

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**Figure 5**

DAP β-lactam-binding site. Superposition of the residues involved in the β-lactam-binding sites of DAP (atoms coloured using standard conventions) and (a) R61 (cyan) complexed with cefotaxime [14]. The 7-ACA group and the acyl moiety of the inhibitor are orange and green, respectively. The black dashes represent the putative stabilisation of the amino group of β-lactam compounds by the γ loop of DAP. (b) Class A β-lactamase from *B. licheniformis* (green) [14] and (c) class C β-lactamase from *E. cloacae* P99 (orange) [14]. Helix α2 and strand β3 of DAP are shown in grey. In order to improve clarity, (a–c) are not represented in exactly the same orientation. The figures were produced using the programs MOLSCRIPT [44] and Raster3D [45].
orientation as cephalothin and cefotaxime in R61 and that their free amino group may be stabilized by interaction with atoms O81 and O82 of Asp481 and the mainchain carbonyl group of Ala482, provided by the γ loop (Figure 5a). Therefore, on the one hand, the presence of the γ loop in the active site of DAP considerably limits the size of potential ligands and, on the other hand, it compensates for the absence of the substrate-binding threonine residues in R61.

In most of the characterised class A β-lactamases, an arginine residue is present at position 244 [17,25]. The α carbon of this residue is situated on the β4 strand and its long sidechain points in the direction of the cavity. Arg244 has been proposed to participate in the binding of the carboxylic acid group of β-lactams. This residue has no evident counterpart in the class C β-lactamase and R61 structures. In DAP, however, Arg295 is in the same location as Arg244 of class A β-lactamases (Figure 5b). The backbone atoms, as well as the Cβ atoms of both residues, are superimposed whereas the guanidinium group of Arg295 is not oriented toward the active centre. However, there is an empty space available to allow a rotation along the Cβ–Cγ bond, thus placing it in a position equivalent to that of Arg244 (Figure 5b). Another interesting feature of Arg244 is its implication in the mechanism of β-lactamase inactivation by suicide substrates (clavulanate and sulbac- tams) [17]. Despite the presence of the arginine residue, the inactivation of DAP by clavulanate and sulbactam is several orders lower than for class A β-lactamases, resembling more the values observed for class C enzymes. With 4 mM clavulanic acid, the rate constants are ~0.0018 s⁻¹ for blinding more the values observed for class C enzymes. With

β backbone atoms, as well as the Cβ atoms of both residues, are superimposed whereas

the general base in the acylation step, the identity of the general base in the acylation step remains controversial, with both Glu166 and Lys73 being potential candidates (see [17] for a detailed discussion). Nonetheless, it is quite clear that Glu166 is an essential residue in the catalytic mechanism of class A β-lactamases. It has no equivalent in PBPs and class C β-lactamases. Rather, in R61 and in the class C β-lactamase from E. cloacae, the lysine residue of the first conserved element (SXXK) and the Tyr150 of the second element (YXN) have respectively been proposed to act as general base in both the acylation and deacylation step, depending on their position and environment in the catalytic site [18].

In the DAP structure, the corresponding residues Lys65 and Tyr153 are situated at 2.9 Å and 2.84 Å, respectively, from the hydroxyl group of Ser62 (Figure 6a), and are therefore in a good position to abstract a proton from the catalytic serine. However, at neutral pH, these sidechains are expected to be protonated and the environment should thus decrease their pKa values by at least 4–5 pH units to enable them to remain unprotonated around neutral pH values. We observed three possible ways for abstracting the proton of the active-site serine residue of DAP. On the one hand, and as observed in the R61 structure where His298 has been proposed to activate the putative base Tyr150, a histidine residue (His287) in the DAP active site is in a good position to interact with Tyr153 (Figure 6a). On the other hand, this tyrosine and the Nδ2 atom of Asn155, which are both hydrogen bonded to Lys65, might contribute to lower the pKa of the latter. The important role of the corresponding lysine residue in the catalytic mechanism of R61 and β-lactamases has been highlighted by mutagenesis experiments. Alternatively, although the DAP Ω loop has a conformation different from that of class A β-lactamases (Figure 3), an acidic residue (Asp225) is in a position equivalent to that of Glu166 (Figure 5b). Its sidechain is not directed towards the catalytic residues but the environment allows a rotation around the Cα–Cβ bond. After such a rotation, the Asp225 sidechain can be positioned similarly to that of Glu166 in class A β-lactamases relative to the conserved serine and lysine residues. Furthermore, a water molecule present near the catalytic serine hydroxyl group, could mediate the interaction between Asp225 and Ser62.

In conclusion, the structural analysis of DAP reveals several residues that are potentially able to act as a general
base in the catalytic mechanism. Further investigation will be necessary to ascertain their respective roles.

The distances separating atoms common to the active sites of β-lactamases A and C, and R61 have been reviewed and compared [30]. Distances separating the Oγ atom of the catalytic serine and atoms of the surrounding conserved residues are of importance for approaching the catalytic mechanism as they help in identifying the residues that might act as the general base. One of these, called the F distance, which spans the oxyanion pocket, distinguishes the class A enzymes from the others [4]. This distance is determined in part by the contact of the β3 strand with the residue immediately upstream of the reactive serine. Class C β-lactamases and PBPs usually have either glycine or alanine at this position whereas class A β-lactamases predominantly have sidechains similar to, or larger than, that of alanine, such that a tilt is created in the β3 strand. The increased F distance in class A β-lactamases has been proposed to allow an optimised interaction between the C6 β-acylamido group of a β-lactam and the β3 strand, so as to favour binding of β-lactams over ß-ß-peptides [30]. In the structure of DAP the residue preceding the catalytic serine is a cysteine (Cys61) and this results in an increased F distance, as in class A β-lactamases. The importance of the nature of this sidechain in DAP, which is itself not in direct contact with the substrate, was confirmed by mutagenesis studies. However, despite this expanded F distance and a number of similarities between the active sites of DAP and class A β-lactamases, the former enzyme preferentially binds ß-ß-peptides. Therefore, the properties of DAP seem to bring the above hypothesis into question, although it should be remembered that the binding of substituted penicillins and cephalosporins is in part restricted by the presence of the γ loop of the C domain protruding into the active site of DAP.

As in ß-ß-peptidases, the turnover of β-lactams by DAP is not significant. There is, however, a major difference. In the former, the turnover is due to the high stability of the covalent acyl–enzyme adduct, which is often rather easily formed. Although the interactions have not been studied in detail, it seems that DAP is not, or very slowly (see the case of clavulanic acid), acylated by β-lactams. In this respect, it will be interesting to compare the structure of DAP with that of a ‘penicillin-resistant’ PBP when the latter becomes available.

The comparison of the topology and the catalytic geometry of domain A of DAP with that of a ß-ß-carboxypeptidase and class C and A β-lactamases confirms the broad
anatomical similarities suggested by sequence comparisons. Compared to the latter, the different substrate- and β-lactam-specificities in DAP are probably due to the presence of the γ loop of domain C protruding into the active site of the enzyme. In this aminopeptidase, domain A provides the catalytic machinery whereas domain C confers the specificity. Domain B probably maintains domain C in a good position to interact with the catalytic domain.

Previous analysis of X-ray crystallographic structures indicated that class A and class C β-lactamases may have evolved from bacterial DD-transpeptidases (for reviews, see [4,18]) but the mechanism of the loss of peptidase activity during evolution remains unclear. A possible mechanism has been proposed for class A β-lactamases involving an increase in the tilt of strand β3 by steric contact with the Ω loop [4]. The analysis and comparison of amino acid sequences and known structures of class C and class A β-lactamases led to the conclusion that these enzymes evolved independently in parallel paths, rather than sequentially along the same path [4].

Biological implications

The emergence of antibiotic-resistant pathogenic bacteria raises a worrying clinical problem. It has been caused by the intensive use of antibiotics, of which β-lactam compounds are the most widely used. These act by inactivating the bacterial DD-transpeptidases, also called penicillin-binding proteins (PBPs), that are involved in the synthesis of the bacterial cell wall. One of the strategies developed by bacteria for escaping these lethal compounds is the synthesis of β-lactamases that hydrolyse the β-lactam ring, thereby avoiding further reaction with the PBPs. β-Lactamases have been proposed to have evolved from an ancestral PBP. The insight into this evolution is of interest in understanding the mechanism of bacterial resistance toward antibiotics.

A new PRP, namely D-aminopeptidase (DAP), has been purified from *Ochrobactrum anthropi*, an emerging nosocomial pathogen. DAP is specific toward peptides with a free D-amino acid at the N terminus. The N-terminal part of its sequence has been shown to share about 25% identity with the Streptomyces R61 DD-carboxypeptidase and the class C β-lactamases, and contains the three motifs conserved in all serine β-lactamases.

We solved the structure of DAP at atomic resolution. DAP folds into three domains: a catalytic domain (domain A) that is highly homologous to serine β-lactamases, and two C-terminal domains (domains B and C). Our study reveals that a loop of domain C, which protrudes into the catalytic site of the enzyme, is probably responsible for both substrate- and inhibitor-specificity. Furthermore, despite a low degree of sequence identity, DAP shows high structural homology with both class A and class C β-lactamases, notably in the catalytic, substrate- and inhibitor-binding sites. Nonetheless, DAP is not readily acylated by β-lactam compounds, an observation that can be explained by a poor positioning of the β-lactam carbonyl group relative to the active-site serine hydroxyl group.

Materials and methods

Expression and purification of DAP

The gene encoding DAP has been cloned from *O. anthropi* SCRC C1–38 and the protein produced in *E. coli* using the pUC18 expression system. After cell lysis, a first purification step was performed by ammonium sulphate fractionation of the clarified extract. The 60–95% saturation precipitate was redisolved in 10 mM potassium phosphate buffer, pH 7, containing 0.1 mM EDTA and 1 mM dithiothreitol (DTT). The solution was loaded onto a Q-Sepharose column which a linear NaCl gradient (0.2–0.4 M) was applied. The fractions containing DAP eluted around 0.35 M NaCl and were concentrated and filtered through an AcA-34 column (20–400 kDa MW range) using the same buffer supplemented with 0.3 M NaCl. The fractions containing the pure protein were pooled and dialysed against 50 mM Tris, pH 8.0, containing 10 mM DTT, 0.1 mM EDTA and 1 mM Na2PO4.

Starting with 3 l of culture, ~45 mg of pure protein was obtained with a purification yield of 55%. The protein was concentrated to 9 mg/ml by dialysis against dry PEG 20,000, and stored at 4°C.

Crystallisation and preparation of heavy-atom derivatives

Crystals of DAP were grown at 21°C using the hanging-drop vapour diffusion method by mixing protein solution and precipitant solution in a 1:1 ratio. Crystals were grown using a protein concentration of 9 mg/ml in 10 mM Tris pH 8, 10 mM DTT, 0.1 mM EDTA and 1 mM sodium azide. The drops contained 2 µl of protein and 2 µl of reservoir solution that contained 30% PEG 400 and 0.1 M sodium acetate, pH 5. Bipyramidal crystals appeared within two days and were large enough for data collection within a week, reaching a maximum length of 0.9 mm.

Platinum and xenon heavy-atom derivatives were prepared by soaking crystals at 21°C in a solution containing 30% PEG 400, 0.1 M sodium acetate, pH 5, and 5 mM K2PtCl4 for 15 h, and by bathing crystals in xenon [31] at a pressure of 10 bar.

Data collection

The crystals belong to space group P412121 with unit-cell parameters \(a = b = 82.86\) Å, \(c = 204.65\) Å. The asymmetric unit contains one molecule and the solvent content is 61.4%.

The native and the xenon derivative data sets were collected at 4°C at the LURE Orsay synchrotron outstation using a Mar Research imaging plate detector on beamline DW32 (\(\lambda = 0.97\) Å) and D41A (\(\lambda = 1.375\) Å), respectively. The platinum derivative X-ray diffraction data set was collected at room temperature on a MacScience DIP2030 image plate system, using CuKα radiation. The latter was produced by a Nonius FR591 rotating anode generator equipped with a double mirror X-ray optical system and running at 100 mA, 45 kV. All the diffraction data were auto-indexed, processed, scaled and merged using DENZO and SCALEPACK from the HKL package [32] (Table 1).

Multiple isomorphous replacement phasing and map quality improvement

The structure was solved by the multiple isomorphous replacement (MIR) method using two heavy-atom derivatives (platinum and xenon [31]). Heavy-atom positions were located by difference Patterson maps and difference Fourier maps using the CCP4 package [33]. Heavy-atom positions, occupancies and isotropic B factors were refined with the program SHARP [34] (Table 1). The initial MIR phases were extended to 1.9 Å and the initial density map was improved by density modification using
related reflections. †RCullis = root mean square (rms) lack of closure/rms average intensity obtained from multiple measurements of symmetry-Rms deviation from ideality

the Protein Data Bank (entry code 1EI5).

GRASP [43], MOLSCRIPT [44] Raster3D [45] and CHEM DRAW [46].

Calculations relating to model validation, model analysis and structural

are 16.3% and 19.2%, respectively, for all data within the 15–1.9 Å reso-

Gaussian bulk-solvent correction was applied. The final R factor and Rfree

ARP [37] was used to add water molecules automatically. Five percent of

likelihood-based refinement cycles employing the program REFMAC [36].

Model building and refinement

The WarpNtrace procedure of ARP [38] was used to attempt automatic
modeling. We used the standard protocol [39] with the ‘big cycle’ being iterated six times. At the end, ARP was able to automatically build 483 of the 520 residues in seven different mainchain fragments. The longest chain, close to the N terminus, contained 242 residues. All chains were docked into the sequence and 296 sidechains were completely built. At this stage, manual fitting into SIGMAA-weighted electron-density maps [39] was initiated using TURBO-FRODO followed by maximum-likelihood-based refinement cycles employing the program REFMAC [38]. ARP [37] was used to add water molecules automatically. Five percent of the observations were set aside for cross-validation analysis [40] and a 2-

Gaussian bulk-solvent correction was applied. The final R factor and Rfree are 16.3% and 19.2%, respectively, for all data within the 15–1.9 Å resolu-
tion range. The results of the refinement are summarised in Table 1.

Calculations relating to model validation, model analysis and structural
alignment were carried out using PROCHECK [41] and TURBO-FRODO [42]. Drawings were generated with the programs TURBO-FRODO [32],
GRASP [43], MOLSCRIPT [44] Raster3D [45] and CHEM DRAW [46].

Accession numbers

The coordinates and structure factors of DAP have been deposited in the Protein Data Bank (entry code 1EI5).

Acknowledgements

This work was supported by the Belgian Program of Interuniversity Poles of Attraction (IUAP/PAI N° P4/03). HR is a research fellow of the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (IWI), LF and MD are fellows of the Fonds pour la Formation à la Recherche dans l’Industrie et l’Agriculture (FRIA), and SJ is Research Associate of the Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium).

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