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D-Amino Acid Residues in Peptides and Proteins

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ABSTRACT **We have investigated the D-amino acid residues present in Protein Data Bank (PDB) entries, categorizing them into "real" D-residues and artifacts. In polypeptide chains of more than 20 residues, only a single instance of a "real" D-residue, other than those deliberately designed or engineered, was found. This example was the result of a slow chemical epimerization process. Another 12 designed D-residues were found in these longer polypeptide chains. Smaller peptides of 20 or fewer residues contained 479 "real" D-residues, the majority in various gramicidin, actinomycin, or cyclosporin structures. We found 148 PDB entries with "real" D-residues and a further 186, in which all apparent D-residues are artifacts. Investigating the (ϕ, ψ) preferences of the "real" D-residues, we found that the region around (−60°, −45°) was almost completely unoccupied, even though it is not formally disallowed. We link the low propensity to occupy this region with the α-helix destabilizing properties of D-residues. Proteins 2003;50:563–571.**

Key words: Protein Data Bank; epimers; chirality; stereoisomerism; D-configurations; secondary structure

INTRODUCTION

Although not normally found in natural proteins, the D-enantiomers of amino acid residues have some well-known properties that often make their incorporation in designed peptides and proteins advantageous. From a structural point of view, their characteristic regions of allowed Ramachandran space make D-residues particularly suitable for stabilizing turns, for instance, in the (i + 1) position of a type II′ or (i + 2) position of a type II β turn.[1,2] From a functional point of view, their unnatural stereochemistry often means that compounds containing D-residues are much more resistant to enzyme-catalyzed breakdown than natural peptides, a property of considerable pharmaceutical and microbiologic importance. Indeed, a number of antibacterial and antitumor compounds contain D-amino acid residues.[3,4]

The genetic code allows proteins to be composed from an alphabet of 20 building blocks, 19 L-amino acids, and the achiral glycine. Because there is no codon for a D-amino acid, the incorporation of any D-residue into a natural biosynthesized protein involves either a posttranslational modification[5–7] or a chemical process.[8]

In order to understand more about D-residues, it is useful to survey all those incorporated in Protein Data Bank (PDB)[9] entries. Because it is to be expected that many apparent D-residues are probably artifacts of the refinement of the X-ray or NMR structures, we have carried out a substantial survey of the PDB to identify the "real" D-amino acid residues.

METHODS

We made a thorough investigation of the entries in the PDB with the assistance of the program suite Procheck[10,11] (version 3.5), and the databases PDBREPORT[12,13] and PDBsum,[14,15] by means of three independent searches. First, we used Procheck to calculate the improper dihedral angle ζ for all residues in the April 2002 release of the PDB. This dihedral angle is defined by the atoms CA-N-C-CB, and should take values around +34° and −34° for L- and D-amino acid residues, respectively. We wrote a script to parse the Procheck output (.rin) files and record as possible D-amino acids all instances of ζ lying between −18° and −52°, respectively; this range corresponds to a tolerance of ±15° plus the modest variation in standard ζ values between residue types. Second, with the assistance of Elmar Krieger (University of Nijmegen), we searched the PDBREPORT database for occurrences of the phrase "Wrong hand" against a C-α atom in the "Chirality deviations" section of the report. This indicates a negative value of ζ and hence a putative D-residue. In this second search, we investigated every model for each NMR structure, whereas in the Procheck search, we examined only the first given model. The PDBREPORT search only identifies D-residues that have been given the standard identifier in the PDB entry (e.g., D-alanine as ALA). We therefore also carried out a third search for D-residues in which a modified name was used (e.g., D-alanine as DAL), using the "Het Groups listing" in PDBsum. Taken together, we believe that these three searches represent as exhaustive a protocol as is reasonably possible. Initially, all possible D-residues identified in one or more of the three searches were retained for further consideration.

Having obtained a pool of putative D-residues, it was then necessary that we separate them into "real" D-residues and "artifacts." A real D-amino acid was indicated when we believed that X-ray crystallography or NMR

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TABLE I. The 492 Putative "Real" D-Residues in the PDB Analyzed by Chain Type, Local Configuration, and Experimental Method

	LDL configurations			Non-LDL			All configurations		
Polypeptide chain types	X-ray	NMR	Total	X-ray	NMR	Total	X-ray	NMR	Total
Gramicidin	96	72	168	0	0	0	96	72	168
Actinomycin	1	10	11	68	0	68	69	10	79
Cyclosporin	48	4	52	0	0	0	48	4	52
Others ≤20 residues	13	9	22	104	54	158	117	63	180
Designed >20 residues	0	10	10	1	1	2	1	11	12
Not designed >20 residues	1	0	1	0	0	0	1	0	1
Total	159	105	264	173	55	228	332	160	492

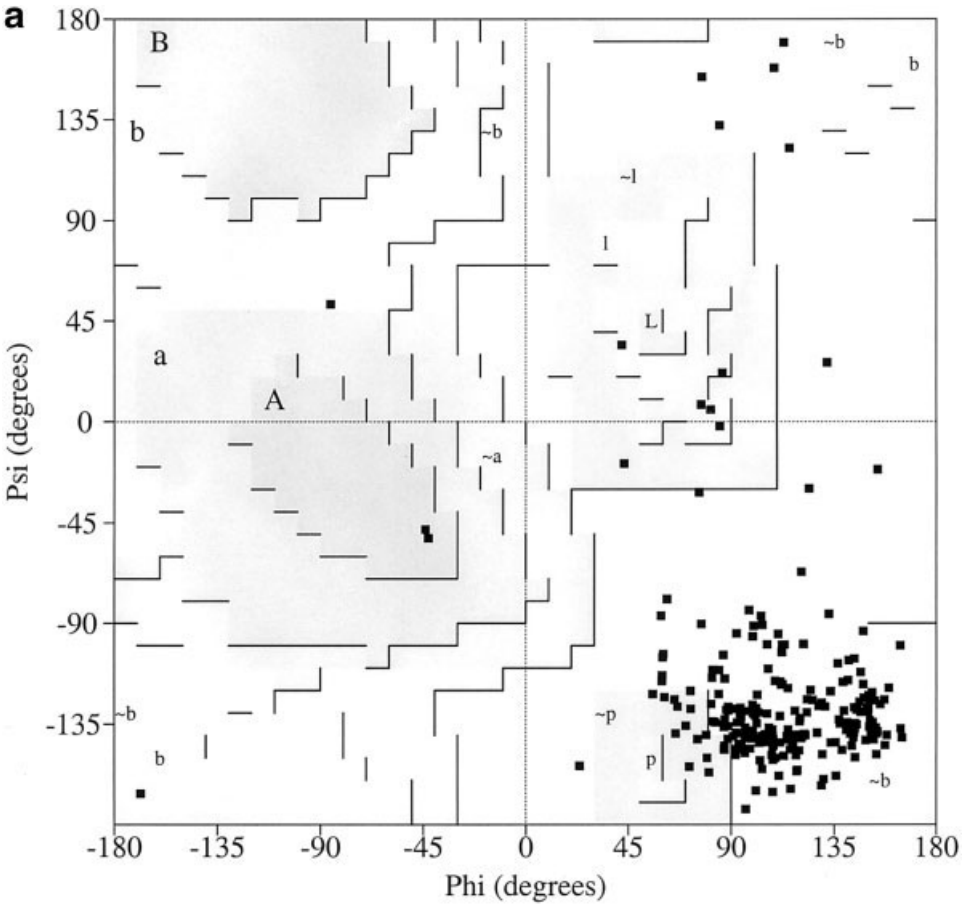

Fig. 1. The (ϕ, ψ) angles of the 264 "real" D-residues found in LDL local configurations: (**a**) plotted on a conventional Ramachandran diagram, (**b**) plotted on an inverted Ramachandran diagram in which the shaded regions correspond to formally allowed regions for a D-residue in a DDD local configuration.

experiments showed beyond reasonable doubt that the given residue adopts a D-conformation, and that the structure in this region had been reliably determined. In almost all cases, the D-residue was deliberately synthesized or engineered into the structure. If our interpretation of the experimental results did not indicate a D-conformation (as in PDB entries 1d7t and 1jzp), then it was excluded, regardless of the original authors' annotations.

An artifact is almost always either a polite way of describing what appears to us to be an error or a D-residue in a theoretical model structure. To identify artifacts, we carried out a number of tests. First, any putative D-residue with a ζ value differing by more than 15° from the expected value was considered an artifact. Although the Procheck search already excludes residues with such distortions, a large proportion of residues so discarded from the PDB-REPORT search were in fact close to flat ($\zeta \approx 0°$). Possible D-residues were also immediately rejected if they came from NMR structures with inconsistent chirality between models, or if they were features of an "averaged" NMR

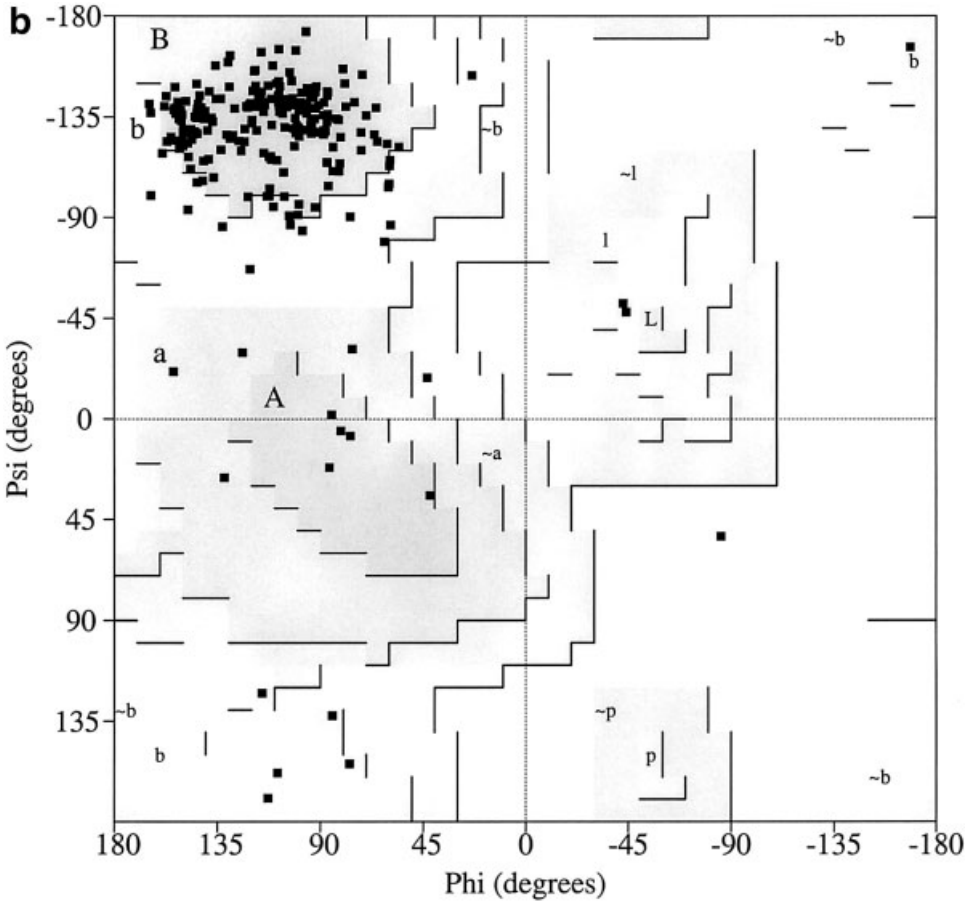

Figure 1. (Continued.)

structure that were not present in the underlying models. All apparent D-residues in theoretical model structures without experimental data were also considered artifacts, as were those in structures whose overall stereochemical parameters, as indicated by PDBREPORT and Procheck, suggested that they probably contained significant errors. The next stage in the determination of a residue's status was to examine the PDB file header. In many cases, this either confirmed that the authors believed the D-residue to be genuine, in which case we accepted it, unless it failed any of the other tests, or that the D-residue was thought to be the result of an error (typically, but not always, noted in a CAVEAT statement). In 10 cases, it was necessary to consult the original literature in order to decide whether the D-configuration should be considered genuine, a positive identification being required in the article. Where we found D-configurations of modified or unnatural amino acids, these were noted but not included in the statistics or analysis. We do not expect that the coverage of these in our search will be as exhaustive as that for the 19 canonical chiral amino acids.

Having separated the pool of possible D-amino acids into real D-residues and artifacts, we further identified among the D-residues those that are both preceded and followed by natural configurations (i.e., L-amino acids or glycine). As a shorthand, we call these LDL local configurations.

We carried out corresponding searches of the Cambridge Structural Database[16] (CSD; April 2001 release), identifying D-amino acid residues, and especially those in LDL local configurations, in the crystal structures of small polypeptides and closely related molecules. For searches of conformational space, the so-called "generous" Procheck[10,11] definitions of the α, β, α-left, and ϵ regions of Ramachandran space were used. When analyzing Ramachandran plots for D-residues, definitions such as "inverted alpha," "inverted beta," and so on, were used; these correspond to the normal definitions when the transformation $(\phi, \psi) \rightarrow (-\phi, -\psi)$ is applied. This transformation is appropriate, because the same reflection or inversion operations that interchange L- and D-configurations map ϕ to $-\phi$ and ψ to $-\psi$. The use of the "mirror image" (ϕ, ψ) angles allows the conformations of D-residues to be visualized against the background of the conventional Ramachandran plot.[17–19] In doing this, however, it is important to remember that the "allowed regions" correspond to those for a D-residue in an all-D-polypeptide, and not necessarily to those for an isolated D-residue in an otherwise all-L-polypeptide.

RESULTS

The PDB searches yielded 492 "real" D-residues distributed among 148 separate PDB entries. For this purpose,

we counted occurrences in separate chains of the same structure as "different." Examples in NMR structures were counted (once) if all the given models were in agreement as to the chirality. The 148 entries containing at least one "real" D-residue contrast with 186 PDB entries in which (all) the putative D-residues were discarded as probable artifacts. These results are listed in full on a web page.[20] Two features are immediately apparent. First, the D-residues in the PDB are almost always found in fairly short-chain polypeptides, with only 13 occurrences in chains of more than 20 residues, and just 1 in a chain longer than 42 residues. Second, the raw data are dominated by a few specific peptides, with 168 of the 492 D-residues being found in gramicidin structures, 79 in actinomycin, and 52 in cyclosporin. These data are shown in Table I.

The 264 D-residues found in LDL local configurations are particularly interesting. They allow us to investigate the conformational space that would be available to a D-residue within an otherwise natural protein or peptide environment. Figure 1(a) shows the areas of Ramachandran space occupied by these residues. The shaded areas, of course, represent the allowed regions for L-amino acids in LLL local configurations. The extensive biases in this particular set mean that the exact populations of each region are unlikely to be meaningful, but the range of conformations occupied is. Figure 1(b) shows the same data superimposed on the allowed regions for a D-residue in an all-D-polypeptide. There are two principal occupied regions, with the most populated one loosely centered around (+120°, −135°), and the other in the α-left region around (+60°, +45°). When inverted in the origin, as seen in Figure 1(b), these two areas correspond to the familiar β and α allowed regions for L-amino acids. We refer to these two regions as "inverted β" and "inverted α," respectively. We note that the "inverted α-left" region around (−60°, −45°) in Figure 1(a), which corresponds to the torsion angles that a D-residue would need to take up to avoid disrupting a conventional α-helix, is almost completely unoccupied, despite corresponding to a formally allowed (analogous to α-left) area of Figure 1(b).

We have also collated a smaller, representative data set intended to minimize the biases due to the similarity of many D-residue–containing molecules. For this purpose, all duplicate chains of identical sequence were removed from the larger data set of 264 residues except for one case (sandostatin), in which there are clearly two separate conformational families. This representative data set contains 40 unique residues and its (ϕ, ψ) map (see Fig. 2 and Table II), shows a cluster around (+90°, −135°) in the "inverted β" region. With use of the Procheck[10,11] definitions of the α and β regions, there are 30 residues in the "inverted β" region, eight in the "inverted α" region, and one in the "inverted α left" region (D-Phe A12 in PDB entry 1 goe). There is one outlier in a disallowed[21] region (D-Phe P2 in PDB entry 1mcf[22]). Six of the eight "inverted α" D-residues and 11 of the 30 "inverted β" D-residues are involved in at least one β or γ turn as defined by Promotif[23] (version 2.0, using the DSSP algorithm of Kabsch and Sander[24]); see Table III. Some residues are involved in more than one overlapping turn: overall, we find that these 17 residues are part of 20 β turns and 4 γ turns. This illustrates the well-known propensity of D-residues within mostly L-proteins and peptides to stabilize turns.[1,2] Analysis of the β turns shows that 16 of the 20 turns do not satisfy the criteria[23] for any of the standard turn types and are hence designated as type IV. Of the 23 nonturn residues, 1 (D-Phe A12 in PDB entry 1 goe) is in an α-helix, 8 are in β strands, and 13 are in nonstructured (coil) regions. The remaining D-residue is listed in Table II as 3_{10} helix; this assignment of D-Trp 4 in PDB entry 2soc is made by Promotif[23] on the basis of the local hydrogen bonding pattern, but the residue is in the ϵ region of the Ramachandran plot (and the "inverted β" region of the inverted plot).

The CSD search identified 120 D-amino acid residues in LDL local configurations. Their (ϕ, ψ) angles are shown in Figure 3, the distribution being broadly similar to that found in the PDB. There are 98 residues in the "inverted β" region and 22 in the "inverted α" region; the "inverted α left" region is completely unoccupied. In contrast, a similar CSD search revealed that of 565 central L-amino acid residues (the definition being strictly applied to exclude glycine as the central residue) in LLL conformations, there were 28 (5%) in the α-left region, 277 in the α region, 253 in the beta region, 4 in what Procheck[10,11] defines as the ϵ region, and 3 in disallowed regions. Next, we established whether the difference between the (in one case, inverted) α-left populations in these two surveys (28/565 and 0/120) is statistically significant. Chi-square analysis gave a probability $p = 1.3\%$ of observing such a result under the null hypothesis of a common underlying distribution. Thus, we reject the null hypothesis and conclude that propensities for the (in one case, inverted) α-left conformation occurring are significantly different for LDL and LLL local configurations. This observation lends support to the idea that the conformational constraints on an isolated D-residue in an otherwise all-L-polypeptide destabilize the "inverted α-left" region.

We have categorized each of the 56 distinct PDB molecules (of at least four residues) we found containing real D-residues in terms of their functions (see Table IV). This functional analysis focused on the role of the D-residues, so that for modified versions of all-L-peptides, the original function was ignored unless fully retained or enhanced in the variant (thus, D-Pro melittin was considered a structural probe rather than a toxin, but D-ALA insulin, PDB entry 1bzv, whose function is reportedly enhanced by the mutation, was considered a hormone mimic). The majority (36/56) were pharmaceutical-related, with the other major categories being designed de novo small proteins (7/56) and structural probes (8/56; a category comprising molecules in which D-residues are introduced to investigate or alter peptide structures, excluding drugs and drug candidates).

DISCUSSION

Our analysis of the apparent D-residues in the PDB demonstrated that a substantial proportion were arti-

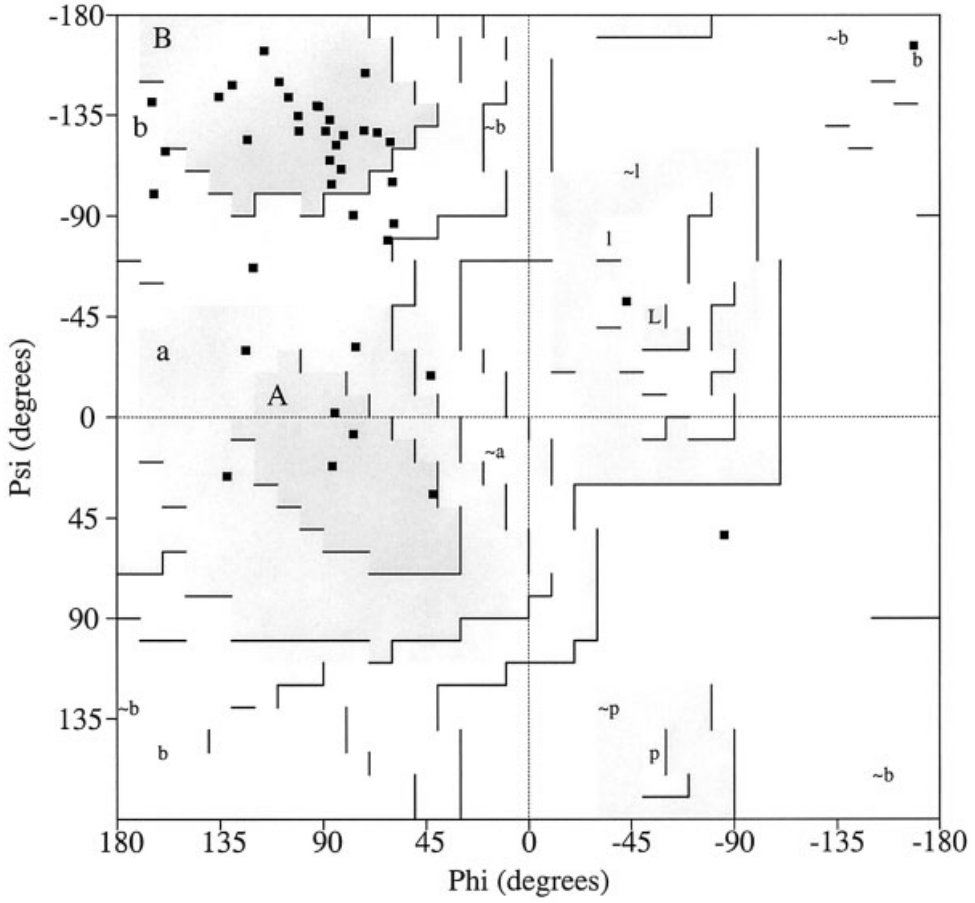

Fig. 2. The (ϕ, ψ) angles of the 40 "real" D-residues forming the representative set obtained by removing duplicates (equivalent residues; see text) plotted on an inverted Ramachandran diagram.

TABLE II. Ramachandran Plot Regions and Secondary Structure Assignments of 40 Representative D-Residues in LDL Local Configurations

	Secondary structure					
Ramachandran	α-helix	3_{10} helix	β-strand	Turn	Coil	Total
Inverted α	0	0	0	6	2	8
Inverted β	0	1	8	11	10	30
Inverted α left	1	0	0	0	0	1
Inverted disallowed	0	0	0	0	1	1
Total	1	1	8	17	13	40

TABLE III. Turn Types Formed by 17 Representative D-Residues in LDL Local Configurations

Position →	*i*	*i* + 1	*i* + 2	*i* + 3	Total
β type I	1	0	0	0	1
β type II	0	0	1	0	1
β type II′	0	2	0	0	2
β type IV	3	6	6	1	16
γ classic	0	4	0	N/A	4

Note: Three of the D-residues are involved two turns, and two are involved in three, as calculated by Promotif[23]; hence, the 24 entries in this table come from only 17 D-residues. The remaining 23 of the 40 representative D-residues are not found in turns.

facts. In X-ray structures, the artifactual D-residues were often located in disordered, mobile, or poorly resolved regions of electron density. To illustrate this, we note that of 138 artifactual D-residues for which suitable data were available, 19 had the highest or equal highest B-factor of any residue in their structures, 73 had B-factors among the highest 10% of those in their structure, and 118 had B-factors in the top half of those in their structure. In many cases, the tetrahedral geometry around the α carbon was highly distorted and far from that expected for either a D- or L-amino acid; 50% of hits in the PDBREPORT search were assigned as artifacts because they had ζ angles more than ±15° from the ideal values. We found 27 NMR structures in which the chirality of a given α carbon varied between models, and also three PDB entries in which some chiralities given in averaged NMR structures did not reflect those of the underlying models (Val 1 and Ser 78 in PDB entry 1pcn; Asn 64 and Phe 66 in PDB entry 1txa; Ala 1 and Ala 3 in PDB entry 1zdb). About 15% (27/186) of all PDB entries containing D-residues that we consider artifacts are in fact listed as "theoretical models" and hence do not directly reflect experimental data. In 10 cases, the PDB, PDBsum, and PDBREPORT entries gave no reason to

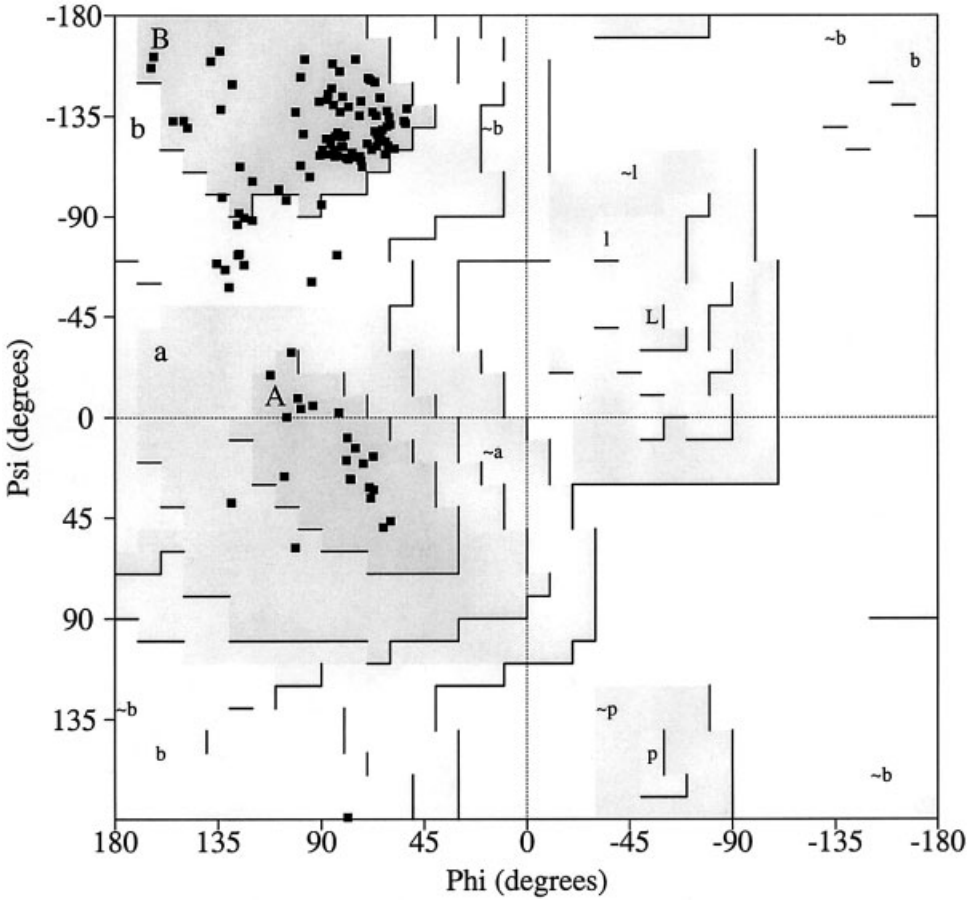

Fig. 3. The (ϕ, ψ) angles of the 120 D-amino acid residues in LDL local configurations found in the CSD, plotted on an inverted Ramachandran diagram.

TABLE IV. Functional Classification of 56 D-Residue–Containing Peptides and Proteins

Functional classification	Number
Total	**56**
Pharmaceuticals	**36**
Antibacterials	$5\frac{1}{2}$
Immunosuppressants	3
Hormone mimics	2
Antitumor agents	$1\frac{1}{2}$
Antithrombins	1
Candidates	23
Others	**20**
Structural Probes	8
De novo designed small proteins	7
Toxins	2
Cell wall peptides	2
D-residue irrelevant	1

Note: Actinomycin D is assigned half to the antitumor class and half to the antibacterial class.

reject the apparent D-residues, but analysis of the primary literature led us to conclude that they were artifacts (PDB entries 1bgl,[25] 1djy,[26] 1dtd,[27] 1ebh,[28] 1ppn,[29] 1ppo,[30] 1tce,[31] 3csc,[32] and 4cts[33]) or a modified amino acid residue (PDB entry lay3[34]). In PDB entries 1d7t and 1jzp, residues listed in the headers as D-tyrosine and D-arginine (DTY A4 in PDB entry 1d7t and DAR A18 in PDB entry 1jzp) have been refined to have ζ angles characteristic of L-amino acids. In the gramicidin structure 1c4d, seven of the 24 D-residues have highly distorted ζ angles. Overall, slightly more PDB entries seem to contain artifactual D-residues (186) than contain real ones (148), whereas 1d7t, 1jzp, and 1c4d (3 PDB entries) probably contain real D-residues but are excluded from our analysis for quality-control reasons.

Putative D-residues in full-length protein chains almost all turn out to be artifacts. The more protein-like structures with real D-residues include 4 designed miniproteins from the Imperiali group[35–37] (containing a total of 7 D-residues), a synthetic analogue of human corticotropin-releasing hormone (PDB entries 1go9 and 1goe), a modified insulin,[38] and the D-Pro melittin structure discussed below. The only example in a full-sized protein occurs in a trypsin structure, namely, residue D-Asp E115 in PDB entry 1an1. This is believed[8] to be formed by a slow chemical epimerization process involving deamidation of the native L-Asn residue followed by stereoselective hydrolysis of the resulting succinimide intermediate. It is listed as "not designed" in Table I, because the D-residue was not

deliberately introduced; however, the D-residue is not expected to be found in the native protein.

Hung et al.[17,18] have published structural studies of a synthetic racemic protein, DL-monellin. Their work suggests that DL-monellin forms an almost centrosymmetric structure, with some minor structural differences between the D-monellin molecules and the mirror images of the L-monellin ones in space group P1. A recent survey of the CSD reported a number of observations of racemates crystallizing in noncentrosymmetric space groups, with small differences between the D-entiomer and the mirror image of the L-form.[39] A small proportion of these noncentrosymmetric small-molecule racemate crystal structures (17/649) were in space groups (such as P1) with no mirror-like symmetry operation.[39] However, the only structure from Hung et al.'s DL-monellin work currently (August 2002) in the PDB is of DL-monellin refined in the centrosymmetric P-1 space group, with an asymmetric unit consisting solely of L-monellin. D-monellin is generated by an inversion symmetry operation. Thus, although the experiments were carried out on DL-monellin, the PDB entry (1krl) itself contains only L-amino acid residues. The monellin structure therefore does not appear in the present study. We also note that D-monellin contains no LDL local configurations (most of the local configurations are DDD, and there are no Gly-X-Gly residue triplets in its sequence).

Clear-cut experimental evidence of α-helix destabilization is available in the case of the introduction of a D-proline residue into melittin, a 26-residue helical polypeptide. This illustrates the effect of a D-residue on an α helix when the "inverted α-left" region is substantially destabilized, as in the case of D-Pro, which strongly favours ϕ angles around +60° (L-Pro conformations are fairly tightly clustered in the range $\phi = -63 \pm 15°$).[40] The normal melittin structure (PDB code 2mlt[41]) has 23 of its 24 nonterminal residues in helices, as defined by Promotif,[23] and all 24 in the α region. The introduction of a single D-Pro near the middle of the sequence (this version has PDB code 1bh1) causes a substantial loss of helicity covering 10 residues, illustrated in Figure 4, so that only 13 residues are in helices. Though 21 of the 24 residues remain in the α region, they are now much less tightly clustered; 2 residues are now in the β region, and D-Pro has an "inverted β" (disallowed for an L-residue) conformation. D-Pro melittin is an extreme case, because the ϕ-angle preferences of D-Pro make it a very strong helix breaker.[42]

The paucity of "inverted α-left" D-residue conformations in our PDB searches and their complete absence in the CSD search of LDL sequences suggest that this region of the Ramachandran plot, which would need to be occupied by a D-residue in an otherwise all-L α-helix, is unfavorable for a single D-residue flanked by L-residues. The destabilization caused by D-residues in α-helices is very probably related to their low propensity to populate the "inverted α-left" region of (ϕ, ψ) space.

The helix-destabilizing properties of D-residues have been studied experimentally by Krause et al.[42] Their work, based on substitution of a pair of D-residues into an amphipathic α-helical peptide, showed D-Pro to be the strongest helix breaker of the 19 D-amino acids. In contrast, D-His and D-Asp were found to be weaker helix breakers than glycine. Chen et al.[43] carried out a conceptually similar set of experiments, substituting singly the L- and D-configurations of each residue into the nonpolar face of another amphipathic α-helical peptide. They noted that the D-configurations of the β-branched amino acids Ile, Val, and Thr were strong helix destabilizers. Fairman et al.[44] also investigated the helix-destabilizing properties of D-Ala as a single D-residue in an α-helical peptide. They found D-Ala to be slightly more destabilising than Gly and noted the likelihood of steric clashes in a right-handed α-helix between the β carbon of D-Ala (i) and the carbonyl oxygen atoms of residues (i) and ($i - 1$). As well as destabilising α-helices, D-residues have been found experimentally to produce a similar effect in β sheets. Krause et al.[45] found that double D-amino acid substitutions significantly disturbed the β-sheet structure of a designed 26-residue peptide.

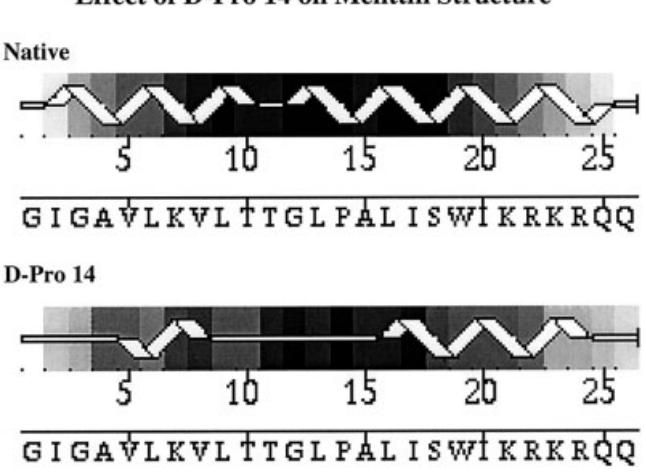

Fig. 4. Effect of the change Pro 14 → D-Pro 14 on the secondary structure of melittin. The upper panel shows the secondary structure of native melittin (X-ray structure of chain A of PDB entry 2mlt[41]) and the lower panel, that of D-Pro 14 melittin (NMR model 1 from PDB entry 1bh1). Darker backgrounds indicate more buried residues.

CONCLUSIONS

We find that "real" D-residues are rare among longer polypeptide chains (20 residues or more) formed from mostly L-amino acids in the PDB and, with a single exception, are the deliberate result of molecular design and protein engineering experiments. Thus, apparent D-residues in proteins are almost always either designed or are artifacts of the X-ray or NMR refinement process. There are many more "real" D-residues in the structures of shorter polypeptides with those in the PDB dominated by multiple structures of gramicidin, actinomycin, and cyclosporin.

Investigating the (ϕ, ψ) preferences of the "real" D-residues, we found that the region around (−60°, −45°) is almost completely unoccupied, even though it is not for-

mally disallowed. We found zero occupancy here for peptide structures from the CSD. We link the low propensity to occupy this "inverted α-left" region with the α-helix destabilizing properties of D-residues. This destabilization has been observed directly by experimentalists,[42,44] as has a similar destabilization of β-sheet secondary structures.[45]

Although analysis of our representative data set confirms the propensity of D-residues to promote β-turn formation, we find that a large majority of the β turns formed do not fall into any of the standard turn types. Thus, although the suggestions that D-residues stabilize the $(i + 1)$ position of a type II′ or $(i + 2)$ position of a type II β turn are useful guidelines for molecular design, they present an idealized view of the structural properties of D-amino acids.[1,2] The functional classification of 56 molecules containing D-residues illustrates how the key properties associated with D-residues (first, the ability of explore and stabilize otherwise unusual conformations, and second, proteolytic stability) are widely used in a pharmaceutical context. The conformational properties of D-residues make them a popular resource in designing structural probes and particularly in the de novo design of small proteins.

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