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Conformational limitations of glycylsarcosine as a prototypic substrate for peptide transporters

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Abstract

Peptide transporters are present in all species to absorb the small peptides that occur ubiquitously as products of proteolysis. The broad substrate specificities of these systems allow them to be exploited therapeutically for delivery of peptidomimetic drugs in microbes and man. To this end, glycylsarcosine is currently used as a standard substrate for assaying peptidomimetic transport by peptide transporters. However, in this study we find it is unsuitable as a general substrate, based on assays of its transport by model bacterial peptide transporters and computer-based conformational analysis of its structure. Of the two generic transporters for di- and tripeptides, exemplified by Dpp and Tpp in *Escherichia coli*, only Dpp can transport glycylsarcosine. The explanation for this finding came from molecular modelling, which indicated that glycylsarcosine can adopt only a restricted range of conformers compared with typical dipeptides, and that of the conformers with a *trans* peptide bond, the majority have the specific psi (ψ) and phi (ϕ) backbone torsion angles needed for molecular recognition and transport by Dpp but none possessed ψ and ϕ torsions required for recognition by Tpp; moreover, 38% of its conformers have *cis* peptide bonds that are not substrates for any peptide transporter. Thus, using glycylsarcosine as substrate in competition assays with compounds that typically form conformers recognised by both types of peptide transporter will underestimate their transport. These findings have implications for assays of oral availability of peptidomimetic drugs such as β-lactams, ACE inhibitors and anti-viral compounds, for which glycylsarcosine is routinely used.

Keywords: Conformational analysis; Drug delivery; Intestinal transport; Molecular modelling; Molecular recognition; Oral absorption; Peptidomimetic

1. Introduction

Peptide transporters exist in Nature to move the small peptides that result from protein hydrolysis across membranes but they also offer an attractive route for delivery of therapeutic peptidomimetics [1–8]. In microorganisms, peptide permeases mostly absorb nutrient peptides produced in their extracellular environment but, in addition, various organisms synthesise antimicrobial peptide analogues that gain access to their intracellular targets through these permeases [1,2,5,7,8]. The analogous systems present in the intestine are there to absorb the products of protein digestion [1–4] but they can also be exploited for oral uptake of a variety of drugs, e.g., to treat hypertension, antiviral agents and β-lactam antibiotics [6–8]. Similar systems occur in other animal tissues and organs as well as in plants and insects [1,5,7].

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The peptide transporters that are best characterised at a molecular and biochemical level are those in bacteria, exemplified by the two archetypal permeases for dipeptides and tripeptides (Dpp (SwissProt P23847) and Tpp (SwissProt P77304)) and that for tri- and higher oligopeptides (Opp (SwissProt P23843 and P76829)) [5,9]. To be able to explain their individual substrate specificities and to design compounds optimised for absorption by one or other transporter, it is necessary to understand the molecular basis for their ability to recognise their natural peptide substrates. This information is provided by descriptions of the individual molecular recognition templates (MRTs) for Dpp, Tpp and Opp [10–13]. The main features involved in defining each MRT are: (i) N-terminal α-amino and C-terminal α-carboxyl groups; (ii) backbone torsion angles ψ, ϕ and ω; (iii) chiral centres at α-carbons; (iv) N–C distance between terminal amino and carboxylate groups; (v) side chain torsion angles, χ; (vi) hydrogen-bond donor and acceptor properties of peptide bond atoms; and (vii) charge fields around terminal amino and carboxylate groups. For the model bacterial peptide permeases, their MRTs were derived using correlations between the results of conformational analyses on a range of di-, tri-, and oligopeptides and measurements of their binding and transport by each permease [10–13]. Although several of these features are shared by all peptide conformers, variations in ψ and ϕ are critical determinants of the individual specificities of each peptide transporter. For example, Dpp-type peptide transporters recognise conformers with ψ of +140° to −175° whereas Tpp-type peptide transporters are specific for ψ of −50° to −85° or +50° to +85°; both types of transporter recognise conformers with ϕ of −50° to −95° or −130° to +175°. These particular backbone conformations impose specific geometries on the charged N-terminal amino and C-terminal carboxylate groups, which are important for ligand binding [10–12]. Definition of the MRTs for these three peptide transporters showed that they complement each other in their substrate specificities, in that each recognises separate, specific classes of peptide conformers so that acting together they maximise absorption of all small peptides [10–13]. Thus, the specificities of these generic peptide transporters have evolved interactively, driven by the selective pressure of the typical repertoire of peptide conformers that arise wherever proteins are hydrolysed. Their substrate specificities are independent of the protein architecture of the transporter, which may comprise a single, membrane-bound, multi-domain protein energised by a proton motive force, e.g., Tpp or a multi-protein, membrane assembly with a separate protein for peptide recognition and binding, e.g., as with Dpp [14].

Because substrates must adopt appropriate backbone conformations to be recognised by one or other peptide transporter, one can evaluate whether or not a compound is a potential substrate for a particular peptide transporter by consideration of the conformers it forms in solution, which can be evaluated by conformational analysis. Amongst the many peptide analogues that have been tested for transport, peptides with *N*-methylated peptide bonds, e.g., glycylsarcosine (GSar; G-N-methyl-G), have been shown to be transported by animal, plant and microbial systems, although in all these systems GSar is transported markedly less well than any normal dipeptide [1,2,5,15,16]. Because these analogues such as GSar are also particularly resistant to hydrolysis by peptidases they can be accumulated intact, overcoming the problem of extensive cleavage commonly seen with normal peptide substrates. Consequently, when such cleavage is a problem, e.g., with intestinal villi, GSar has become the prototypic substrate used in competition assays to evaluate transport of peptidomimetic drugs by intestinal, renal and other peptide transporters [2,4,7,8,17,18].

In this paper, we have examined GSar for its suitability as a standard substrate for use in such transport assays. Conformational analysis shows that it adopts a limited range of conformers that restrict it to being a substrate for Dpp-type transporters and not for Tpp-type transporters. This theoretical prediction was corroborated by transport assays with specific peptide permease mutants of *Escherichia coli* and peptide binding studies, which showed GSar to be transported by Dpp but not by Tpp. These findings may compromise the use of GSar for assessing absorption of peptidomimetic compounds, particularly for compounds that exist mainly in conformations recognisable by a Tpp-type rather than a Dpp-type transporter.

2. Materials and methods

2.1. Conformational analysis of peptides

Peptides were modelled in their zwitterionic form using a random search procedure in which all torsions were allowed to vary; SYBYL 6.5 software (Tripos, St Louis, MO) running on a Silicon Graphics Octane workstation was used, essentially as described [10–13]. The computed conformers for each peptide were ranked according to energy, and by comparing the energy of each with that of the minimum energy conformer, the percentage of each form was calculated using a Boltzmann distribution. For each conformer, its ψ, ϕ and ω torsion angles were measured, together with other relevant features such as N–C distance [10–13]. For each compound, ψ and ϕ torsional space for the effective peptide unit was divided into 36 10° sectors and for all conformers with particular ψ–ϕ combinations their individual percentages were aggregated. The collection of conformers for any compound could then be displayed using a three-dimensional pseudo-Ramachandran (3DPR) plot that relates percentage of conformer to its ψ and ϕ angles. This plot conveniently shows the extent to which the conformer profile of any compound matches the torsional requirements of the MRT for each peptide transporter [10–13].

Energy profiles for peptides were calculated using grid search conformational analysis, with ω maintained at $\pm 180°$ and ϕ at $-75°$ and with ψ allowed to vary from $-170°$ to $\pm 180°$ in 10° increments.

2.2. Assays of peptide transport and binding

Transport studies were carried out using *E. coli* K-12 peptide permease mutants described previously [9]: strain PA0410 (*opp*, *tpp*), which has only a functional Dpp, and strain PA0333 (*opp*, *dpp*), which has only an active Tpp. Transport of [^{14}C]GSar by strains PA0333 and PA0410 was measured essentially as described [9]. With this substrate, which is peptidase resistant, the amino acid efflux seen with normal peptides is not a problem for transport assays [5]. Briefly, exponential-phase bacteria (10 ml 10^9 cells ml^{-1}) were incubated with GSar (0.1 mM) in 50 mM phosphate buffer (pH 7.2) with 0.2% (w/v) D-glucose, 1 ml samples were removed periodically over about 12 min, filtered through glass-fibre membranes, and washed twice with 5 ml phosphate-buffered saline before drying and counting for retained radioactivity. Controls were performed without cells or GSar. In addition, GSar transport was assayed fluorimetrically, as was the transport of other dipeptides, as described previously [9].

Binding of [^{14}C]GSar (specific radioactivity 56.8 mCi mmol^{-1}) (Cambridge Bioscience) and [^{14}C]GL (specific radioactivity 20 mCi mmol^{-1}) (Amersham) to dipeptide-binding protein (DppA) was carried out as described previously [9]. Briefly, samples of purified DppA (20 μM, 29 μg) freed from endogenous ligands were incubated with varied amounts of [^{14}C]GL (25 μl, 1–600 μM) or GSar (25 μl, 50 μM–1 mM) at 37°C for 30 min, before addition of 0.9 ml ice-cold, saturated ammonium sulphate solution; samples were filtered using polycarbonate membranes in a Millipore manifold and washed three times with 2.0 ml of the ammonium sulphate solution before counting for retained radioactivity. Controls were carried out in which DppA or ligand were omitted.

3. Results

3.1. Conformational analysis of dipeptides

In previous studies, a random search method has been shown to be optimal to determine the repertoire of conformational forms adopted by charged dipeptides in water [10–13]. This approach was used here to analyse GSar; for comparison, modelling was done of the analogues GG and GP, the latter often being used as an alternative substrate to GSar, and of the standard dipeptide AA. For each peptide, separate random searches of 1k and 5k iterations were performed and results were combined; the total number of unique conformers found for GSar, GP, GG and AA were 37, 15, 55 and 18, respectively. The energies, torsional values and N–C distances for the twelve main conformers of each peptide are shown in Table 1. For GSar and GP, only those conformers with ψ angles of about +160° to $-175°$ match the requirements of an MRT, whereas all conformers of AA and to a lesser extent those of GG match well [10–12]. Minimum energy conformers of GSar and

Table 1
Conformational parameters for main conformers of AA, GG, GP and GSar

Conformer	Energy (kcal mol^{-1})	Boltzmann	Percent	ψ	ω	ϕ	N–C (Å)
AA1	2.40	1.00	22.52	165.0	178.4	−65.1	5.300
AA2	2.57	0.76	17.12	165.4	−179.1	−159.1	6.122
AA3	2.67	0.65	14.57	164.3	178.6	51.3	5.056
AA4	3.02	0.37	8.29	73.1	179.6	−65.6	5.332
AA5	3.03	0.36	8.11	73.7	−178.0	−159.8	5.410
AA6	3.08	0.33	7.50	−62.6	178.9	−66.0	4.656
AA7	3.15	0.29	6.66	−61.5	−178.5	−159.2	5.119
AA8	3.19	0.28	6.22	73.3	179.8	51.4	4.683
AA9	3.37	0.21	4.69	−62.5	179.3	51.4	5.277
AA10	3.67	0.13	2.87	−63.0	178.4	−82.5	4.647
AA11	4.39	0.04	0.89	147.8	−2.7	−74.2	4.339
AA12	4.99	0.01	0.33	104.4	0.6	−61.3	3.355
GG 1	2.61	1.00	12.40	−179.3	179.3	−65.1	5.247
GG 2	2.61	1.00	12.40	179.3	−179.4	65.1	5.248
GG 3	2.71	0.85	10.61	−78.5	178.5	−66.1	4.637
GG 4	2.71	0.85	10.62	78.5	−178.5	66.1	4.636
GG 5	2.83	0.70	8.75	78.1	179.8	−65.2	5.366
GG 6	2.83	0.70	8.75	−77.9	−179.8	65.4	5.365
GG 7	2.93	0.60	7.41	179.9	± 180	179.9	6.166
GG 8	3.07	0.47	5.85	77.8	−179.6	179.4	5.384
GG 9	3.07	0.47	5.85	−77.8	179.6	−179.3	5.383
GG 10	3.28	0.34	4.20	−77.7	179.6	−179.3	5.367
GG 11	3.51	0.23	2.89	172.1	−1.5	−75.9	4.612
GG 12	3.51	0.23	2.89	−172.1	1.5	75.9	4.613
GP 1	9.03	1.00	21.09	171.7	−0.3	−78.6	4.644
GP 2	9.06	0.94	20.05	171.2	−179.7	−75.7	5.413
GP 3	9.40	0.55	11.64	−168.9	178.7	−51.9	5.112
GP 4	9.64	0.37	7.92	−161.0	−1.3	−55.0	4.463
GP 5	9.66	0.36	7.58	−94.6	179.8	−75.6	4.770
GP 6	9.70	0.34	7.11	−95.0	−0.2	−78.3	4.382
GP 7	9.70	0.33	7.07	173.3	± 180.0	−76.5	5.399
GP 8	9.91	0.24	5.09	103.8	2.8	−70.0	3.464
GP 9	10.00	0.21	4.38	105.2	−1.7	−56.1	3.332
GP 10	10.14	0.16	3.47	95.6	178.7	−51.8	5.389
GP 11	10.34	0.12	2.51	91.2	−176.4	−80.8	5.540
GP 12	10.75	0.06	1.30	−88.0	175.1	−48.4	4.693
GSar 1	4.39	1.00	14.80	−168.9	0.0	80.6	4.623
GSar 2	4.39	1.00	14.80	168.9	0.0	−80.7	4.623
GSar 3	4.47	0.88	12.91	173.0	−178.6	69.7	5.168
GSar 4	4.47	0.88	12.91	−173.1	178.6	−69.7	5.169
GSar 5	4.60	0.71	10.44	−170.3	± 180.0	88.2	5.514
GSar 6	5.22	0.26	3.80	−96.9	± 180.0	−73.9	4.736
GSar 7	5.27	0.24	3.49	−95.8	0.3	−78.9	4.376
GSar 8	5.27	0.24	3.49	95.8	−0.3	78.9	4.376
GSar 9	5.37	0.20	2.97	−106.1	−0.4	62.2	3.350
GSar 10	5.37	0.20	2.97	106.0	0.4	−62.3	3.350
GSar 11	5.39	0.20	2.91	95.2	178.8	−68.9	5.432
GSar 12	5.40	0.19	2.85	97.4	−179.8	87.9	4.820

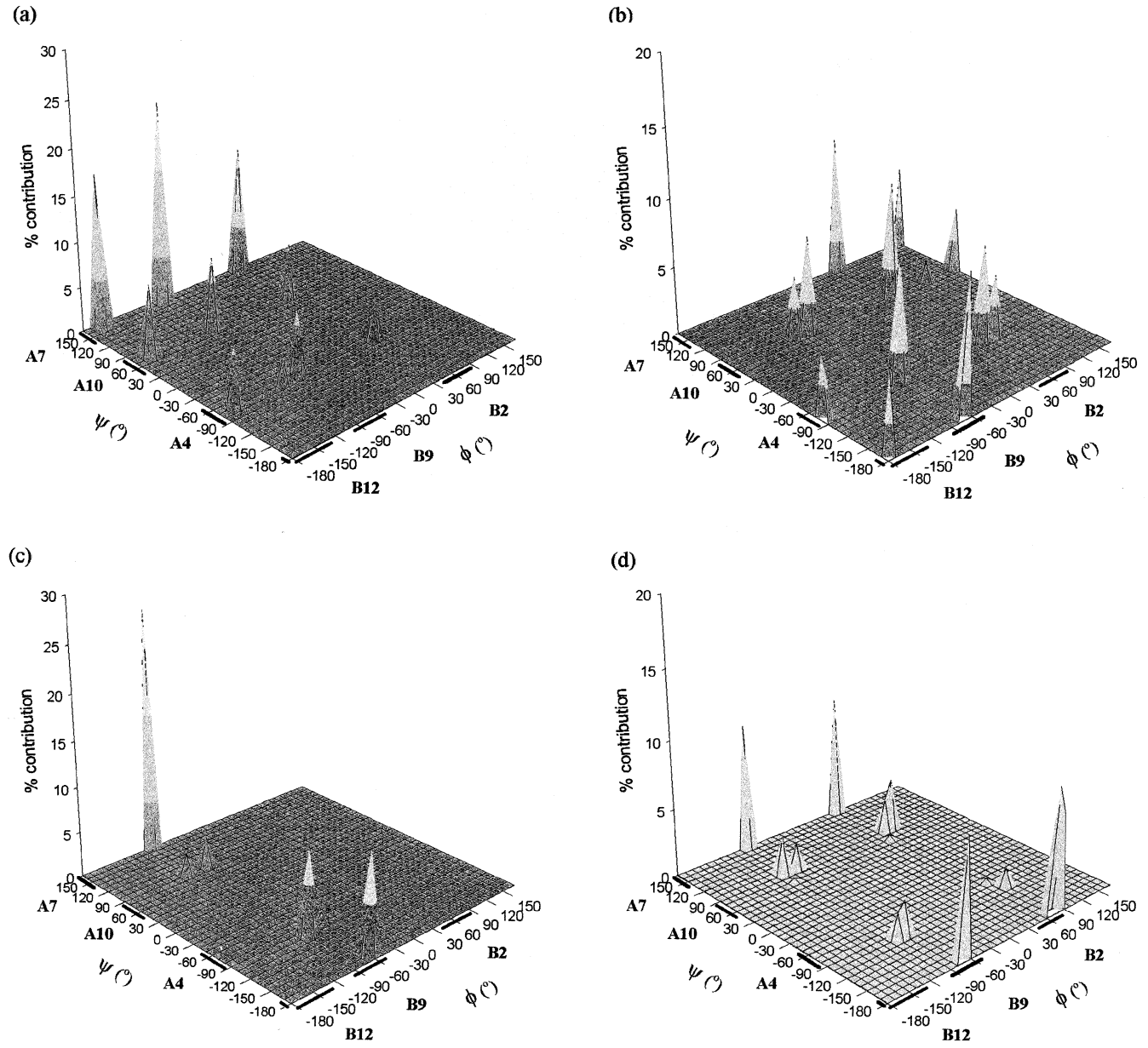

Fig. 1. Three-dimensional pseudo-Ramachandran plots of dipeptides. (a) Alanylalanine, AA; (b) glycylglycine, GG; (c) glycylproline, GP; (d) glycylsarcosine, GSar. For each compound, the percentage of each conformer (excluding those with *cis* peptide bonds) is plotted against its ψ (A1–A12) and ϕ (B1–B12) torsions. Conformers recognised by Dpp-type DT(A7) transporters have A7B9 and A7B12 torsions and those recognised by Tpp-type DT(A4,10) transporters have A4(B9+B12) and A10(B9+B12) torsions; precise values for these torsional angles are given in the text.

GP have *cis* peptide bonds and correspondingly shorter N–C distances. Because only peptides having *trans* peptide bonds can be recognised and transported by peptide transporters [1,2,5,9–13,19,20], *cis* conformers need first to be removed from consideration as putative substrates. The total amounts of conformers computed to exist with *cis* peptide bonds for GSar, GP, GG and AA were 38%, 46%, 14% and 1%, respectively.

Using only the collection of *trans* conformers for each peptide, the percentage of each conformer was plotted against its ψ and ϕ angles to produce a 3DPR

plot (Fig. 1). The plots for these peptides share many features found for all dipeptides [10–13] but each shows unique characteristics. AA is a model dipeptide [9–13] and its ψ and ϕ torsions are typical of most dipeptides (Fig. 1a): ψ torsions are in the ranges +145° to +175°, −70° to −85° and +65° to +85°, referred to as A7, A4 and A10, respectively, and its ϕ torsions occur in the ranges −60° to −70°, −145° to −170° and +50° to +60°, referred to as B9, B12 and B2, respectively [10–13]. Determination of the molecular recognition parameters for bacterial peptide transporters has established that substrates of Dpp-type transporters must possess A7 ψ torsions, whereas Tpp-type transporters are specific for A4 and A10 torsions; both recognise B9 and B12 ϕ torsions but neither can recognise B2 forms [10–13]. With GG, its main conformational forms occupy related ranges to those for AA but the greater backbone rotation uniquely allowed with the small side chains of G results in a much wider spread of ψ and ϕ angles (Fig. 1b). Its A7, A4 and A10 ψ torsions fall approximately within the ranges +140° to −175°, −50° to −85° and +50° to +85°, respectively, and its B9, B12 and B2, ϕ torsions occur approximately in ranges −50° to −95°, −130° to +175° and +40° to +85°, respectively; these deviations from optimal torsions help to explain why it is one of the poorest natural dipeptide substrates for all peptide transporters [1,2,5,9–12]. GP adopts a much more limited range of conformers, with acceptable ψ torsions being restricted to A7 and others falling outside recognisable A4 and A10 ranges, with ϕ torsions being confined to B9 sectors (Fig. 1c). GSar also adopts a restricted conformational range (Fig. 1d). However, whereas its A7 torsions are those of a typical dipeptide its other very minor conformers fall outside the range of recognisable A4 and A10 sectors found in any typical dipeptides [10–12]. The percentages of the *trans* conformers that exist in the recognisable ranges

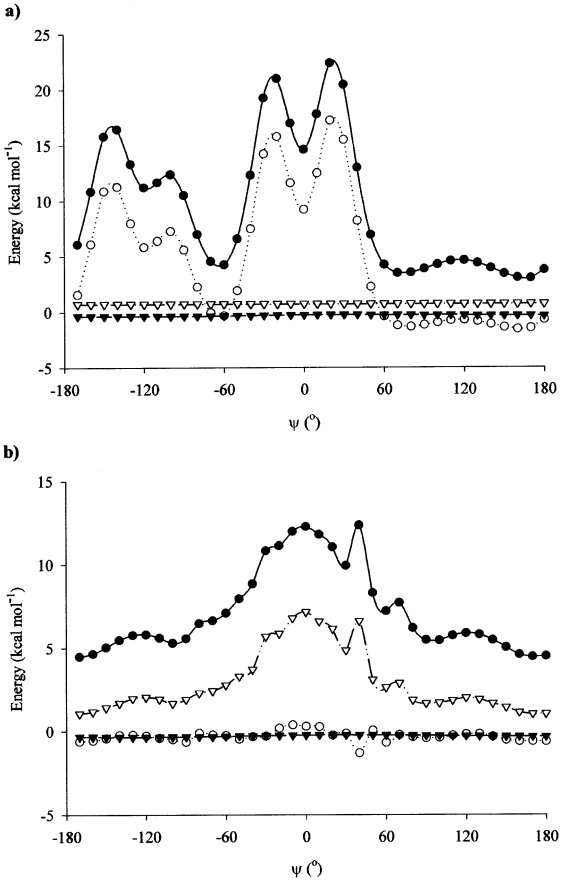

Fig. 2. Energy profiles for AA and GSar computed using grid search. ω (±180°) and ϕ (−75°) torsions were kept constant whilst ψ angles were systematically varied by 10°. Individual energy contributions to the total energy of each conformer were calculated and plotted for AA (a) and GSar (b); total energy (●); van der Waals energy (○); angle bending energy (▽); electrostatic energy (▼).

of ψ and ϕ for these four dipeptides are summarised in Table 2. These results indicate that the conformational forms of GSar and GP appear to restrict these dipeptides to being substrates for Dpp-type but not for Tpp-type peptide transporters [10–12].

The reasons why AA and similar dipeptides adopt

Table 2
Percentages of main conformational forms of AA, GG, GP and GSar recognised by peptide transporters

Peptide	A7 conformers			A4 conformers			A10 conformers		
	B9	B12	B2	B9	B12	B2	B9	B12	B2
AA	22.5	17.1	14.6	10.4	6.7	4.7	8.3	8.1	6.2
GG	10.3	12.3	10.3	8.8	4.9	11.9	11.9	4.9	8.8
GP	26.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GSar	9.4	0.0	8.9	0.0	0.1	0.3	0.3	0.0	0.4

a particular range of conformers [12], whereas GSar (and GP) with a modified peptide bond adopts a more restricted range, becomes clear from consideration of the factors that contribute to the overall energies of the conformers (Fig. 2). Grid searches were carried out in which ψ angles were systematically varied by 10° over the whole of torsional space whilst ω was fixed in a *trans* bond ($\pm 180°$) and ϕ ($-75°$) was kept constant in a B9 torsion. For AA the main determinant of its energy profile is van der Waals energy whereas, unusually, with GSar angle bending is the main contributor, making putative A4 and A10 conformers unstable, high energy forms (Fig. 2).

3.2. Transport of GSar by peptide transporters

Transport of [^{14}C]GSar was measured using mutants of *E. coli* in which only specific peptide transporters were functional [9]. Results for transport by strain PA0410 (*opp*, *tpp*), which has only a functional Dpp, and for strain PA0333 (*opp*, *dpp*), which has only an active Tpp, are shown in Fig. 3. The initial rate of [^{14}C]GSar transport by Dpp was 3.4 nmol min^{-1} mg bacterial protein^{-1}, whereas transport by Tpp was not detectable even at concentrations up to 10 mM (results not shown). Transport of unlabelled GSar by the two strains was also measured using a fluorescence assay; transport by Dpp was 3.5 nmol min^{-1} mg bacterial protein^{-1} and zero by Tpp. Natural dipeptides derived from proteins exist typically with the majority of their conformers shared between the specific forms recognised as substrates by Dpp and Tpp [5,9–12] giving similar rates of uptake by each, e.g., for AA, 28.5, 23.5; GF, 24.3, 14.5; GY, 33.1, 17.4; LW, 35.0, 14.4; and SA, 34.6, 27.1 for Dpp and Tpp, respectively. The ratio of these rates is typically in the range 1.2–2.4; in contrast, for GSar the zero rate of uptake by Tpp makes this ratio infinity.

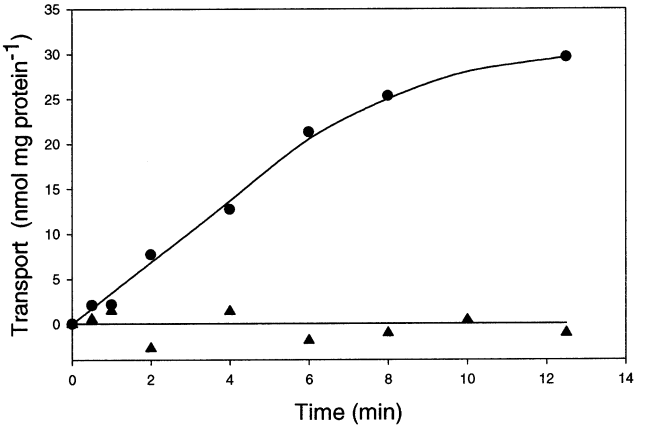

Fig. 3. Transport of glycylsarcosine by specific peptide transporters in *Escherichia coli*. Strain PA0410 (*opp*, *tpp*) has only a functional Dpp system (●) and strain PA0333 (*opp*, *dpp*) has only a functional Tpp system (▲).

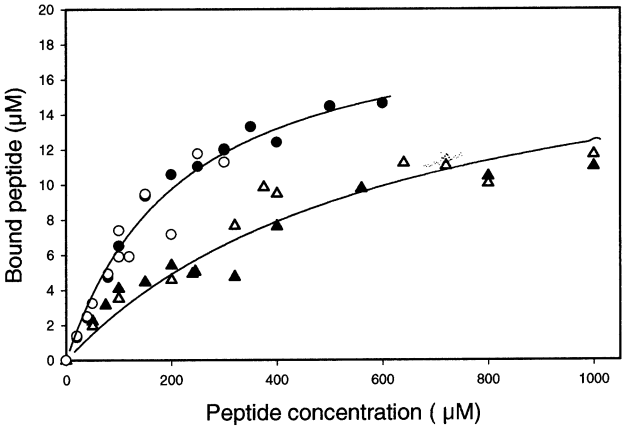

Fig. 4. Binding of dipeptides to peptide binding protein DppA. Curves shown are for two separate binding experiments for glycylleucine (●,○) and glycylsarcosine (▲,△).

3.3. Binding of GSar to dipeptide binding protein

The dipeptide binding protein, DppA, is responsible for the initial recognition of substrates by Dpp and there is a close correlation between binding of compounds to DppA, measured using purified protein in vitro, and their transport through Dpp [5,9–11]. Therefore, this provides an alternative technique to evaluate the bioactivity of the conformer repertoire of GSar. Fig. 4 shows the binding curve for [^{14}C]GSar with DppA, together with that for [^{14}C]GL as a model substrate. Binding constants were estimated to be about 0.6 mM and 0.2 mM for GSar and GL, respectively. GL has been used as a model substrate in the X-ray structure determination of a liganded-DppA complex, in which GL adopted an A7B9 conformation [21], in accord with the predictions based on molecular modelling [10–12].

4. Discussion

4.1. Common structural specificities of peptide transporters

Transporters for components of the peptide pools that result from protein hydrolysis are ubiquitous in Nature, being found in microorganisms, higher and lower plants, insects and animal tissues and organs [1]. Similar substrate specificity features are observed for all these transporters, which may otherwise differ in their complement of proteins, or their modes of energisation or regulation [1–8]. Detailed descriptions of the molecular recognition properties for individual peptide transporters [1–9,22–25] are compatible with these specificities having evolved similarly in response to the structures of the same natural substrates in the peptide pool [10–12].

4.2. Bacterial peptide transporters specific for dipeptides and tripeptides

In bacteria, molecular genetic techniques have established the existence of two main permeases with specificity only for di- and tripeptides, i.e., Dpp- and Tpp-type transporters [1,5,9,14]. Extensive studies have established detailed substrate specificities for these transporters [5,9] and these have been explained in terms of the specific structural conformers adopted by di- and tripeptides in solution [10–12]. From correlating these structure-activity relationships, the individual MRTs for a Dpp- and a Tpp-type transporter have been defined with respect to the backbone torsions, side-chain orientations, N–C distances, etc., of a peptide substrate, and the two transporters found to be complementary in recognising different conformational forms of a peptide [10–12]. Similar MRTs are likely to have evolved for di- and tripeptide transporters in all organisms in response to the common selective pressure provided by the conformer classes found in these peptides [11].

4.3. Classification of peptide transporters

Peptide transporters can be classified by various criteria, and this becomes an important feature when making comparisons between transporters in various organisms. Thus, gene sequences, energy coupling mechanisms, protein number/architecture and substrate specificities can each be used for categorisation but these different criteria may cut across classification schemes. A classification based upon gene sequencing gave five categories [14]: one class being primary transporters (direct use of chemical energy) in the ATP-binding cassette (ABC) superfamily, the other four being secondary transporters driven by a proton gradient. ABC transporters in prokaryotic and eukaryotic microorganisms, e.g., Dpp and Opp, comprise five proteins: one (DppA, OppA) being a recognition/binding protein, which may be free as in *E. coli* or membrane-bound as in *Bacillus subtilis*, the other four proteins forming a membrane-bound complex. Analogous mammalian ABC transporters have all five proteins fused together, with the substrate recognition domains being part of the membrane-bound complex [26]. Secondary transporters are members of the proton-dependent oligopeptide transporter (POT) or peptide transport (PTR) family that comprise single proteins typically containing 12 transmembrane domains [14,27,28]. Bacterial Tpp and mammalian PepT1and PepT2 belong to this class [8,9,14,29]. Thus, these ABC and POT transporters have entirely different protein architectures yet all can transport common peptide substrates. Furthermore, other hybrid transporters have been characterised that are composed of combinations of distinct functional modules of both secondary and ABC transporters, indicating that during evolution the combination of integral membrane transport proteins with either a cytosolic ATPase or recognition/binding protein, or both, has resulted in distinct types of transporters [30]. Consequently, gene motifs, protein architecture or energy coupling mechanisms may be permutated in various ways and classifying a peptide transporter as one type or another by these criteria does not necessarily allow extrapolation to definitive conclusions regarding their precise substrate specificities. Classification based on substrate specificity requires results from biochemical assays of transport and binding together with insights into conformer profiles of substrates [9–13], as illustrated by the studies here. However, there is presently no classification scheme based on substrate specificity, which makes it potentially confusing to compare substrate specificities of, e.g., Dpp (or DppA) with PepT1, for their terminologies often de-

rive from other classifications. Thus, using 'Dpp-type' and 'Tpp-type' when referring to transporter specificities as we have here, can unfortunately carry connotations of gene sequences, protein architecture and energy coupling. Consequently, we propose a classification for peptide transporters based on their substrate specificities that includes the length of peptide and the (pseudo) ψ torsional angle(s) of their substrate conformers, which allows their substrate specificities to be compared irrespective of their classifications by other criteria. Thus, by this scheme, the substrate specificity of Dpp and DppA is termed DT(A7) (recognising Dipeptides and Tripeptides with ψ of A7 (+140° to −175°)) and Tpp becomes DT(A4,10) (recognising Dipeptides and Tripeptides with ψ of A4 and A10 (−50° to −85° or +50° to +85°)). As a further extension to these arguments, it is well accepted that transporters for various amino acids may be ABC systems and/or proton-linked systems in microorganisms whilst with the same substrate specificities they may be proton- or sodium-linked secondary transporters in mammalian cells, and, in addition, that many peptidases and proteases can also be conveniently classified in terms of their substrate specificities although their protein architectures and catalytic mechanisms may often be markedly different [31].

4.4. GSar as a substrate of peptide transporters

The broad specificity profile of peptide transporters allows them to recognise peptides with certain structural modifications. For example, peptides with N-methylated peptide bonds are substrates, albeit relatively poor ones, for peptide transporters in microorganisms, plants, intestine and kidney [1–10]. The marked resistance of such peptides to peptidase action has led to the adoption of GSar as a standard substrate, particularly for studies with many mammalian tissues in which natural peptides are extensively hydrolysed [2–4,6,8]. In such studies, radioactively-labelled GSar acts as substrate with transport of other compounds being assessed from their ability to inhibit GSar uptake [6–8,17,18,25]. GP shows analogous resistance to peptidases and it is often used to complement studies with GSar [7,8,17,18]. Consequently, GSar, and to a lesser extent GP, have been adopted as prototypic substrates for studies on oral drug availability. The presumption in their use is that the ability of a therapeutic compound to inhibit their uptake is generally an accurate measure of the compound's own ability to be absorbed by the various peptide transporters present, except for the few compounds that may bind and compete without actually being transported themselves [8].

4.5. Computer modelling of peptide substrates

A molecular modelling approach has been adopted in several attempts to define the structural features needed for substrate recognition by mammalian peptide transporters using a range of natural substrates and analogues that exploit peptide transporters, e.g., dipeptides, β-lactams and other drugs [7,22–25]. In the more recent studies, important structural parameters have been identified that are in broad agreement with results for bacterial peptide transporters derived from modelling natural peptide substrates [10–13]. Given these corroborations between theoretical modelling and empirical, structure–activity relationships, we have here analysed GSar and GP to assess their suitability as model substrates for peptide transporters, whether it be for studies on antimicrobial peptidomimetics or for oral drug delivery. Modelling showed that GSar and GP exist with 38% and 46%, respectively, of their conformers with *cis* peptide bonds (Tables 1 and 2). These theoretical values agree well with published experimentally values: GSar $39 \pm 1\%$ and GP $48 \pm 2\%$ [19,20], endorsing the general validity of the computed repertoire of conformers for these compounds. Brandsch and coworkers demonstrated that PepT1 could only transport peptides with *trans* peptide bonds [19,20] and the same conclusion was reached previously for microbial transporters from consideration of MRTs, which showed that peptides with *cis* peptide bonds fail to match several, critical structural parameters [5,9–13]. Consequently, here we evaluated only those conformers with *trans* peptide bonds as putative substrates and these were displayed as 3DPR plots (Fig. 1). Conformers of GSar and GP exist only with A7 ψ torsions, which when combined with B9 ϕ torsions restrict them to being substrates exclusively for DT(A7) (e.g., Dpp) transporters [10–12]. This expectation was endorsed by results showing that in *E. coli*

GSar could only be transported by a strain with a functional Dpp system, no transport being detectable by Tpp (Fig. 2). Similarly, GSar and GP have previously been shown to compete for uptake of dipeptides by Dpp but not by Tpp ([9–11]; unpublished results). Typically, natural dipeptides are transported through both systems [5,9–12]. A strong correlation exists between transport of a compound by Dpp and its binding to DppA, the recognition protein for Dpp [5,9–12]. Here, GSar was shown to bind to DppA but relatively poorly compared with GL and more typical dipeptides [9–12], reflecting its lowered content of *trans* forms and departure from the ideal parameters for molecular recognition by DppA.

4.6. Number of intestinal peptide transporters

Early studies on intestinal peptide transport addressed the question of the number of different peptide transporters present and Matthews, in a scholarly overview of the area [2], concluded that "...though a very large number of di- and tripeptides of dissimilar structure do compete for intestinal transport there are in fact at least two peptide transport systems in the small intestine", and in this context he cited the lack of reciprocal inhibition (when extrapolated to infinite concentrations) between GSar and other dipeptides such as AA, LL and VV. More recently, the accumulated evidence for the existence of several peptide transporters has been reviewed [8,14], including use of techniques for cloning and identification of peptide transporter genes and proteins and extensive substrate specificity studies aimed at defining the number and nature of peptide transporters involved in oral absorption of peptidomimetic drugs. Fei and colleagues [29] identified the intestinal transporter PepT1 using an expression cloning strategy with *Xenopus laevis* oocytes and screening an intestinal cDNA library for uptake of [^{14}C]GSar: using a hybrid-depletion experiment, they also concluded that PepT1 was the only peptide transporter for GSar in enterocytes, although in such experiments variations in the timing and conditions for mRNA expression do not rule out the occurrence of other peptide transporters [8]. However, their conclusion that PepT1 is the only transporter that can recognise GSar [8,24] would accord with GSar modelling reported here. Furthermore, the fact that PepT1, and the analogous renal transporter PepT2 [8,14], both recognise GSar (and GP), which can only exist as A7 conformers, is prima facie evidence for consideration of their classification as DT(A7) transporters.

The question remains as to the number of di-, tripeptide transporters present in the intestine, kidney (and other organs). Results from competitive transport assays and other studies point strongly towards there being more than one [1,2,8], and analogous studies in other organisms would support this expectation. If any unidentified transporter is a DT(A4,10) type then its identification will require use of a substrate(s) that adopts appropriate conformers with A4 and/or A10 ψ torsions.

4.7. Implications of GSar structure for assays of peptide transport

The results reported here imply that the use of GSar (and/or GP) as substrates in competition assays to assess transport of competitor compounds will be liable to error depending upon the conformer profile of the competitor. For a compound that exists exclusively with the torsional forms A7(B9,B12) that match DT(A7) transporters, e.g., bacterial Dpp, and potentially PepT1 and PepT2, correlation between competitive ability and transport should be good. In contrast, if a compound exists with a significant proportion of its conformers in other torsional forms such as A4,A10(B9,B12) that are recognised by a DT(A4,10) transporter, then correlation will be poor with uptake being underestimated. This conclusion could have significant implications for current procedures to assess oral absorption of therapeutic peptidomimetics by intestinal and renal peptide transporters. Support for this has come not only from modelling natural peptides but also a range of orally active ACE inhibitors and β-lactam antibiotics, which showed them to exist with not only A7 but also with A4 and A10 torsional forms (unpublished results). Further support for the suggestion that Dpp and PepT1 may possess similar DT(A7) substrate specificities has come from the finding that a range of peptidomimetic ACE inhibitors, e.g., enalapril, enaliprilat, lisinopril, etc., bind to DppA and that their relative binding affinities show a similar ranking to that found for their abilities to compete for uptake

of substrates by PepT1 (unpublished results). It still remains to be determined whether any additional intestinal peptide transporter(s) possesses the complementary DT(A4,10) specificity; however, arguing teleologically it would appear likely for that would enhance absorption of the extensive component of peptides produced by protein breakdown in the gut that will exist in conformational forms recognised exclusively by such transporters [10–13]. If peptide transporters in different organisms have not evolved common specificities in response to similar selection pressures, i.e., conformer profiles of their natural peptide substrates, the question arises as to what has provided the driving force for their specificities; looking for answers in the structures of poorly transported, synthetic mimetics that can exploit these systems does not seem to offer the best approach.

The present results highlight the advantages of multi-disciplinary studies of related biological systems across various organisms, exemplified here by ubiquitous membrane transporters that have evolved similar specificities to optimise molecular recognition of common substrates. Our current studies with substrates for peptidases and proteases (unpublished results) indicate that the benefits of such an approach will not be limited to the rational design of therapeutic agents tailored for delivery by peptide transporters.

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