

Structure of gramicidin D–RbCl complex at atomic resolution from low-temperature synchrotron data: interactions of double-stranded gramicidin channel contents and cations with channel wall

M. L. Główka,^{a*} A. Olczak,^a
J. Bojarska,^a M. Szczesio,^a
W. L. Duax,^{b,c} B. M. Burkhart,^b
W. A. Pangborn,^b D. A. Langs^b
and Z. Wawrzak^d

^aInstitute of General and Ecological Chemistry, Technical University, Łódź, Poland,

^bHauptman–Woodward Medical Research Institute, Buffalo, NY, USA, ^cState University of New York at Buffalo, Buffalo, NY, USA, and

^dDND-CAT Synchrotron Research Center, Argonne, IL, USA

Correspondence e-mail: marekgl@p.lodz.pl

Received 16 August 2004

Accepted 5 January 2005

PDB References: gramicidin D–RbCl complex, 1w5u, r1w5usf

Gramicidin D (gD) is a naturally occurring ionophoric antibiotic that forms membrane channels specific for monovalent cations. The crystal structure of the RbCl complex of gD has been determined at 1.14 Å resolution from low-temperature (100 K) synchrotron-radiation data with a final *R* of 16%. The structure was refined with anisotropic temperature factors for all non-H atoms and with partial occupancies for many of them. The asymmetric unit in the crystal contains four crystallographically independent molecules that form two right-handed antiparallel double-stranded dimers. There are seven distinct rubidium-binding sites in each dimeric channel. The occupancy factors of Rb cations are between 0.11 and 0.35 and the total ion contents of the two crystallographically independent channels are 1.59 and 1.22 ions, respectively. Although each channel is 'chemically symmetrical', the side-chain conformations, the distributions of rubidium cations and their binding sites in the two independent channels are not. Cations are 'coordinated' by delocalized π -electrons of three to five carbonyl groups that together with peptide backbone chains form the gramicidin channel walls. The water:cation ratio in the channel interior is four or five:one, and five or six waters separate Rb cations during their passage through the channel.

1. Introduction

Gramicidin D, an oligopeptide synthesized by the soil microorganism *Bacillus brevis* (Dubos, 1939), is toxic to *Staphylococcus* pathogens (Dubos & Hotchkiss, 1941) and Gram-positive bacteria (Hotchkiss, 1944; Gross & Witkop, 1965). Gramicidin forms intramembrane channels that allow monovalent cations to escape from cells, disrupting ion balance (Pressman, 1965). Gramicidin D (gD) consists of six linear pentadecapeptides having Val (Vg) or Ile (Ig) at position 1 and Trp (gA), Phe (gB) or Tyr (gC) at position 11. The sequence of the main component, gramicidin A, is formyl-Val¹-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp¹¹-D-Leu-Trp-D-Leu-Trp-ethanolamine (Sarges & Witkop, 1965). Position 11 is known to influence the duration of channel opening and ion-transport properties (Becker *et al.*, 1991). In commercially available gD, relative concentrations are given for gA, gB and gC but not for Vg and Ig (Fluka; Sigma).

In the early 1970s, two principal models for the structures of the ion-transport form of gramicidin were proposed. The two models, a head-to-head single-stranded helix (HSH; Urry *et al.*, 1971) and an intertwined antiparallel double-stranded double helix (DSDH; Veatch *et al.*, 1974), were later found experimentally by X-ray and NMR techniques. Of several monomeric and dimeric gramicidin forms reported to be

present in solution (Bystrov & Arseniew, 1988; Zhang *et al.*, 1992; Pascal & Cross, 1993; Wallace, 1998), only DSDH forms have been observed in crystal structures. Prior to 1998, five X-ray crystal structures of gramicidin had been reported: three uncomplexed forms crystallized from alcohols, namely methanol (Langs *et al.*, 1991), ethanol (Langs, 1988) and *n*-propanol (Burkhart, Gassman *et al.*, 1998), and two ion-complexed forms (Cs⁺ and K⁺) crystallized from methanol (Wallace & Ravikumar, 1988; Doyle & Wallace, 1997). All five structures were reported to be left-handed antiparallel double-stranded β -helices (DS β H₂) with 5.6 residues per turn in the uncomplexed structures and 6.4–7.2 residues per turn in the cation-complexed structures. Although the nature of the hydrogen bonds differs between the uncomplexed structures and the Cs⁺ and K⁺ complexes, they all contain a common dimeric ribbon linked by 16 hydrogen bonds. The alternating D,L chiral composition places all of the amino-acid side chains on one side of this ribbon. Because of steric crowding, the ribbon coils away from the side-chain covered surface and forms a second chain of hydrogen bonds, thus forming a two-stranded β -barrel. Although there are 14 hydrogen bonds in the second set in both the complexed and uncomplexed structures, the registry is shifted by two residues in the complexed structures. This shift makes the channel diameter larger and the channel length shorter, thus accommodating the presence of ions in the channels of the complexes.

The hydrogen-bonding pattern proposed to satisfy the NMR spectra of gramicidin in methanol is identical to that observed in crystals of uncomplexed gramicidin grown from ethanol, methanol and propanol (Burkhart *et al.*, 1999). However, the hydrogen-bonding patterns and conformations proposed to satisfy the NMR spectra of the ion-complexed form of gA in polar solvent (Arseniev *et al.*, 1985) and in lipids (Lomize *et al.*, 1992) are entirely different to that reported for the crystal structures of the Cs⁺ and K⁺ complexes (Wallace & Ravikumar, 1988; Doyle & Wallace, 1997). There is nearly universal agreement that the active-transport form of gramicidin is right-handed as demonstrated by CD spectra (Killian *et al.*, 1988) and NMR studies (Hu & Cross, 1995). Consequently, neither the X-ray structure of the left-handed uncomplexed form nor the left-handed Cs⁺ and K⁺ complexes (Wallace & Ravikumar, 1988; Doyle & Wallace, 1997) were considered to be relevant to the ion-transport process.

The presence of anomalous characteristics of the reported crystal structures of the Cs⁺ and K⁺ complexes of gramicidin A led us to re-investigate the crystal structures of the complexes. The anomalous characteristics included the facts that (i) diffraction data recorded in the range 2.5–1.9 Å were excluded from the refinement of the K⁺ complex, (ii) the φ and ψ values of four Leu and Val residues in the structure of the K⁺ complex were in unfavorable regions of the Ramachandran plot (Ramachandran & Saisekharan, 1968), (iii) side-chain atoms including tryptophan side chains on two of the gramicidin molecules were excluded from the refinement of the K⁺ complex owing to overlap in the model, (iv) unsolvated Cs⁺ ions were reported to reside on the hydrophobic outer surface of the channels, (v) the same locations within the channel were

Table 1

Crystal data, solution and refinement statistics.

Crystal data for gD–RbCl complex	
Formula of main component (gA)	C ₉₈ N ₂₀ O ₁₆ H ₁₄₄
Weight (Da)	2036
Crystal system	Orthorhombic
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 30.06, <i>b</i> = 31.30, <i>c</i> = 51.69
Unit-cell volume (Å ³)	48634
Formula units <i>Z</i>	16
Size of crystal (mm)	0.5 × 0.4 × 0.3
Linear absorption coefficient (mm ⁻¹)	0.29
Crystallographic data-collection details	
Wavelength (Å)	0.71 (synchrotron)
Detector	MAR CCD 165 mm
No. of images	321
Resolution range (Å)	20.0–1.14
Highest resolution shell (Å)	1.18–1.14
Total No. of reflections	639497
No. of unique reflections	33140
No. of reflections with <i>F</i> > 4σ(<i>F</i>)	29741
<i>R</i> _{merge} (%)	6.7 (33.9)
Data multiplicity	16.9
Completeness (%)	98.7 (97.2)
Refinement statistics and other details	
Restraints	9453
Parameters	3352 (block 1), 3485 (block 2), 6567 (overall)
Final <i>R</i> _{free} (%)	20.2
Final <i>R</i> (%)	16.2
Final <i>R</i> , all data (%)	17.1
<i>wR</i> ²	0.49
Goodness of fit (GooF)	1.19
Δ <i>F</i> (max, min) (e Å ⁻³)	0.47–0.45
<i>w</i>	0.45
Flack parameter	0.02 (0.02)

occupied by Cl⁻ ions in the Cs⁺ complex and K⁺ ions in the potassium complex and (vi) the backbone geometry and details of hydrogen bonding were significantly and inexplicably different in the two complexes. Many of these features are considered to be hallmarks of incorrect structure determinations.

Crystals having the same space group (*P*2₁2₁2₁) and nearly identical unit-cell parameters as those reported by Wallace (*a* = 31, *b* = 32, *c* = 52 Å) were obtained by following the procedures described in her paper. However, structure determination revealed a different structure with gramicidin molecules in a right-handed antiparallel double-stranded β -helix (DS β H_{3R}) form instead of the left-handed form reported by Wallace & Ravikumar (1988).

Seven crystal structures of monovalent cation complexes of gramicidin (CsCl, TiNO₃, KCl, KI, KSCN and RbCl from methanol and ethanol) refined against room-temperature diffraction data (collected on a laboratory area detector) provide detailed information concerning the distribution of ions and water in a single-file channel (Duax *et al.*, 2002, 2003). Monovalent cations and water molecules occupied several sites in the gramicidin channels with partial occupancies and the side chains of some of the peptide residues are disordered. High-resolution structure determinations using synchrotron radiation were needed in order to understand the nature of the ion coordination in the channel and gain insight into the mechanism of ion transport. The first of these high-resolution studies, the RbCl complex, is reported here.

Table 2

Important statistical parameters for the final structure.

(a) Statistical parameters.

No. of non-H atoms (or sites)	
Peptide atoms (VgA assumed)	544
Peptide-atom sites refined (including disorder)	659
All atom sites refined (including partially occupied and alternate conformers)	720
Cations (Rb ⁺) sites	14
Anions (Cl ⁻) sites	12
Water sites	31
Ethanol molecules (atom sites)	1 (4)
R.m.s. deviations	
Bond distances (Å)	0.016
Bond angles (°)	1.0
Torsion angles (°)	1.5
Ramachandran plot statistics (%)	
Residues in most favored regions	34 L residues (inc. Gly), 24 D residues
Residues in additional allowed regions	0
Residues in generously allowed regions	0
Residues in disallowed regions	0

(b) B factors (Å²)

	Average	Minimum	Maximum
Main chains	19.3	11.2	56.5
Side chains	23.1	10.1	53.4
Cations	20.7	12.2	30.2
Anions	30.6	8.9	63.1
Water molecules inside the channels	25.5	16.4	51.6
Water molecules outside the channels	43.7	5.7	45.6
Ethanol molecules	56.5	52.2	61.2

2. Materials and methods

2.1. Crystallization and data collection

Commercially available gramicidin D (Sigma), analytical grade RbCl and ethanol were used in simple batch crystallization. For cryotemperature (100 K) data collection, a single crystal of the gD–RbCl complex was picked up in a fiber loop, immersed in a glycerol/water (3:1) solution for a few seconds, scooped out in another loop mounted on a diffractometer pin and placed under liquid nitrogen into a transportation tank. Diffraction data were collected on beamline 5-ID of the DND-CAT at the Advanced Photon Source, Argonne, IL, USA using a 165 mm MAR CCD detector and were processed with the *HKL* software package (Otwinowski & Minor, 1997). Data were recorded with $\lambda = 0.71$ Å, giving a significant anomalous signal for Rb atoms. Table 1 shows a summary of data collection and structure refinement.

2.2. Structure refinement

The method of determination of the structure of the CsCl complex of gramicidin is described in detail in the paper by Burkhart, Li *et al.* (1998). The right-handed Burkhart model has none of the anomalies present in the Wallace model and our extensive efforts to refine the Wallace model with data collected from our cation complexes have failed. Consequently, the Burkhart structure was taken as the starting model in this study. The model was refined with the *SHELX97* package (Sheldrick & Schneider, 1997) with conjugate-

gradient least squares (CGLS). Anisotropic least-squares refinement with manual adjustment enabled the detection of disorder in many side chains, the identification of additional solvent molecules and ions and refinement of their occupancy factors. The multiple-conformation site-occupancy factors for side chains were refined constraining their sum to be unity. The occupancy factors of Rb⁺ cations, Cl⁻ anions and water molecules in the channels were unrestrained during refinement. Most of the temperature factors of the O atoms of water molecules in the channels were refined isotropically. H-atom positions were refined in riding positions on their parent atoms. Engh & Huber (1991) restraints on bond distances and angles were used in refinements. The data-to-parameter ratio of about 9 was achieved by dividing the structure into two partially overlapping blocks (33 194 data over 3352 parameters in block 1 and 33 194 data over 3485 parameters in block 2), each comprising one independent dimer with its interior and the same interchannel volume (Table 1).

3. Results, their validation and discussion

The final refined model of the gramicidin D complex with RbCl consists of 659 non-H atom positions from two gramicidin dimers (four gD molecules comprising 64 amino-acid residues), 2.8 Rb⁺ and 2.6 Cl⁻ atoms distributed over 14 (Rb⁺) and 12 (Cl⁻) positions, 31 water sites and one ethanol molecule (Table 2) were included. Both the two independent dimers and the gramicidin molecules within each dimer are related by approximate non-crystallographic twofold axes. In the numbering system adopted for the main-chain peptides 1xy (strand 1) and 2xy (strand 2) are intertwined in an anti-parallel fashion and form channel I, while peptides 3xy and 4xy form channel II (Fig. 1). Consequently, Trp211 refers to the 11th amino acid in peptide 2xy of dimer I. The data are of high quality, with r.m.s.d.s. of 0.016 Å for bond distances, 1.0° for bond angles and 1.5° for torsion angles (Table 2). Analysis of the molecular geometry was performed with the *CCP4* suite (Collaborative Computational Project, Number 4, 1994) and *PROCHECK* (Laskowski *et al.*, 1993).

3.1. Heterogeneity of the gramicidin

Gramicidin D is a mixture of gA (~80%), gB (~5%) and gC (~15%) forms (Weinstein *et al.*, 1980). In this study, Tyr residues were only found in one of two strands in each dimeric channel. The possible role of gA–gC heterodimers in the nucleation of gramicidin crystals and the relationship between solvent and heterodimer composition has been discussed elsewhere (Burkhart, Gassman *et al.*, 1998). The percentages of tyrosine and tryptophan residues in position 11 were 23 and 77% in strand 2, and 19 and 81% in strand 4, giving average amounts of Tyr11 in the two channels of 11.5% in channel I and 9.5% in channel II in the crystals. This is within the range given for the composition of the starting material. However, there was no indication of Ile being present in position one of any of the four crystallographically independent strands as demonstrated by the lack of features in a difference Fourier

map at a 1.3–1.5 Å distance from the terminal C atoms of Val1. Also, no trace of the gB component was detected.

3.2. Main-chain and side-chain conformations

The four independent peptide chains have very similar main-chain or backbone conformations and their φ and ψ

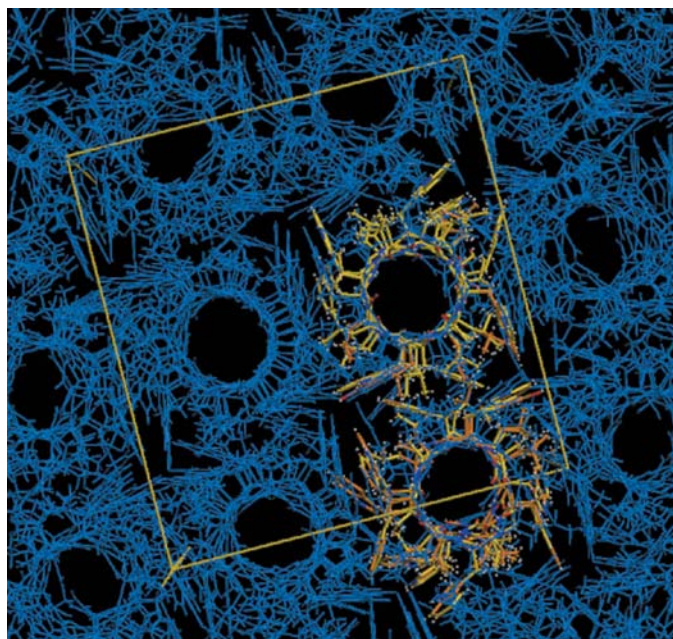


Figure 1
The packing of molecules in crystals of the gD–RbCl complex viewed along [001]. The two crystallographically independent double-stranded antiparallel dimers forming infinite channels in the crystal are thickened. This figure was produced using *Xfit* (McRee, 1999) and *Raster3D* (Merritt & Murphy, 1994).

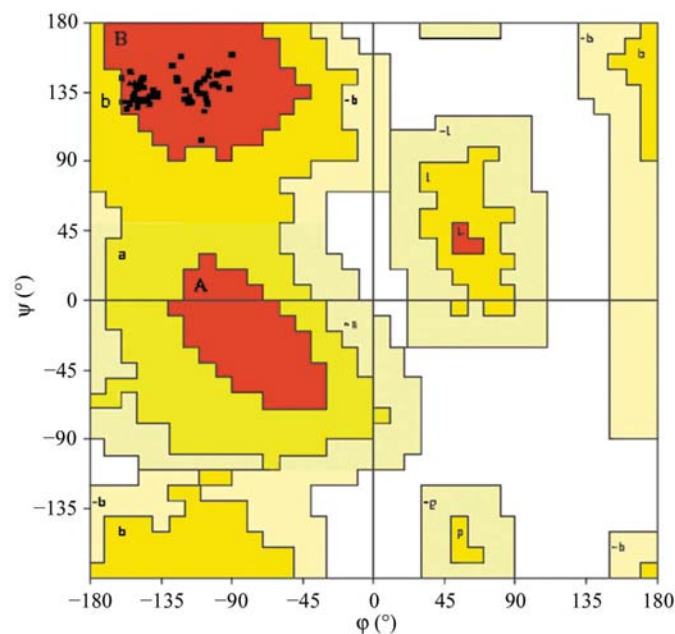


Figure 2
Ramachandran (φ , ψ) plot (Ramachandran & Saisekharan, 1968) assessed by *PROCHECK* (Laskowski *et al.*, 1993) for the studied gramicidin structure. The φ and ψ angles of D-amino acids were converted (reversed signs) to the top left corner.

angles are within the values found in the most favored regions of the Ramachandran plot (Fig. 2), after taking into account the change of signs for the torsion angles of D-amino acids. The maximum positional differences for all residues are in the range 0.05–0.49 Å (Fig. 3).

Examination of the torsion angles (Table 3) also reveals an overall similarity between the two dimers in the asymmetric unit. Torsion angles defining side-chain orientations in strand 1 are similar to those in strand 3 and torsion angles in strand 2 are similar to those in strand 4. However, the two strands forming each dimeric channel (*i.e.* strands 1 and 2) have significantly different side-chain orientations, revealing the asymmetry of the channels. In the case of the largest side chains (Leu and Trp), the same patterns of conformational variation are present in the Cs and Rb complexes (Table 3). Namely 20% of the 48 Val, Leu and Trp residues have twofold conformational disorders, 14 involving the χ_1 angle and six involving the χ_2 angle, with occupancies ranging between 52 and 81% for the more populated form. The greater degree of disorder observed in the RbCl complex (this study) compared with the CsCl complex may be a consequence of the higher resolution and resulting higher accuracy in the RbCl structure determination. Greater variation in the chain orientations for valine residues is observed both when comparing channel I with channel II and when comparing (Table 3) the RbCl complex with the CsCl complex (Burkhart *et al.*, 1999).

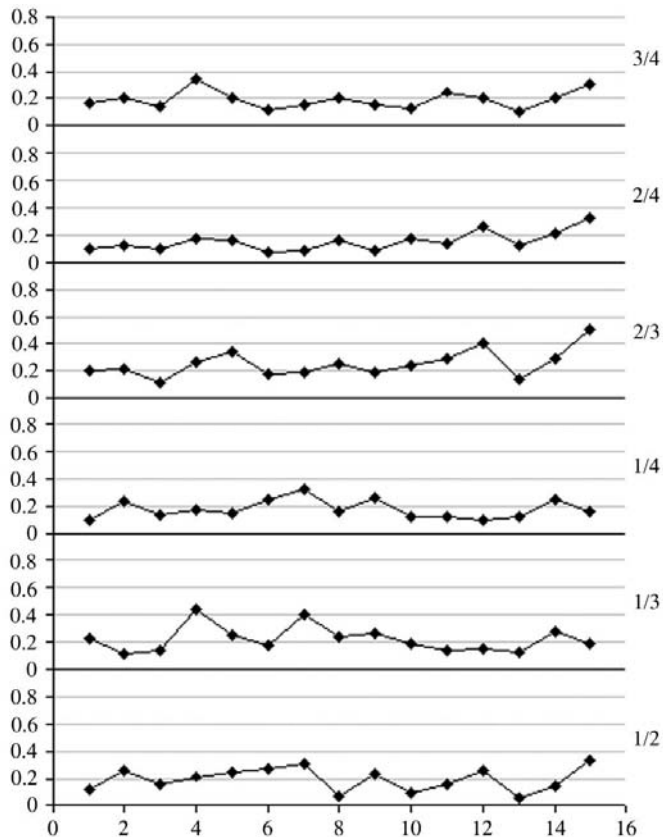


Figure 3
Comparison of main-chain conformations in four independent oligo-peptide strands (except for the hydroxyl groups) shown as the ratio between average positional differences.

Table 3

Populations of side-chain conformations (χ_1) in right-handed double-stranded gramicidin complex crystal structures.

The older *gauche-trans* notation has been used for simplicity. Occupancy (in %) of the χ_1 conformer with the higher population is shown. An asterisk (*) indicates positional disorder for χ_2 . For residues Trp211 and 411 occupancies (given in parentheses) refer to Tyr substitution.

Residue	Strand 1	Strand 2	Strand 3	Strand 4
RbCl-gD (this work)				
Val1	g^+	t	g^+	t
D-Val6	g^+ (77), t	t	g^+ (58), t	t (54), g^+
Val7	g^-	g^+ (57), g^-	g^- (55), g^+	g^+
D-Val8	g^+	t	g^- (61), g^+	g^- (68), t
D-Leu4	g^+ (58), t	g^+	t	g^+
D-Leu10	g^- (54), g^+	g^- (78), t	g^+	g^- (66), g^+
D-Leu12	t	g^- (66), g^+	* t (55)	g^-
D-Leu14	t	t (78), g^+	t	g^-
Trp9	g^-	g^-	g^- (61), t	g^-
Trp11/Tyr	g^-	* g^- (23)	g^-	* g^- (19)
Trp13	t	* g^- (52)	t	* g^- (52)
Trp15	t	g^-	* t (65)	g^-
CsCl-gD (Burkhart <i>et al.</i>, 1999)				
Val1	g^+	t	g^-	g^-
D-Val6	g^-	g^+	g^-	g^-
Val7	t	g^-	g^-	t
D-Val8	g^-	g^+	g^+	g^-
D-Leu4	g^+	g^+	t, g^+	g^+
D-Leu10	g^-	g^-	g^-	g^-
D-Leu12	t	g^-	t	g^-
D-Leu14	t	g^-	t	g^-
Trp9	g^-	g^-	g^-	g^-
Trp11/Tyr	g^-	g^-	g^-	g^-
Trp13	t	g^-	t	g^-
Trp15	t	g^-	t	g^-

Table 4

Occupancies and temperature factors (\AA^2) of cations and waters in gramicidin channels as found in this study.

	Rb ⁺		H ₂ O	
	Occupancy	Temperature factor (<i>B</i>)	Occupancy	Temperature factor (<i>B</i>)
Channel I	0.21	26.5	0.98	39.7
	0.21	14.3	0.55	16.8
	0.19	16.0	0.55	16.1
	0.20	12.0	0.46	17.7
	0.35	9.9	0.56	17.9
	0.26	17.4	0.33	15.5
	0.17	21.3	0.56	14.2
			0.48	18.0
			0.66	16.8
			0.29	14.1
			0.22	19.2
Sum	1.59 (7 sites)		5.64 (11 sites)	
Average		16.7		18.7
Channel II	0.17	13.3	0.59	16.9
	0.22	16.3	0.74	16.4
	0.15	14.0	0.25	15.5
	0.16	11.5	0.80	15.1
	0.11	18.6	0.52	15.2
	0.15	14.9	0.84	20.7
	0.27	13.7	0.68	18.7
			0.48	17.8
			0.84	16.9
			0.40	21.5
			1.00	75.5
Sum	1.23 (7 sites)		7.14 (11 sites)	
Average		14.6		22.7

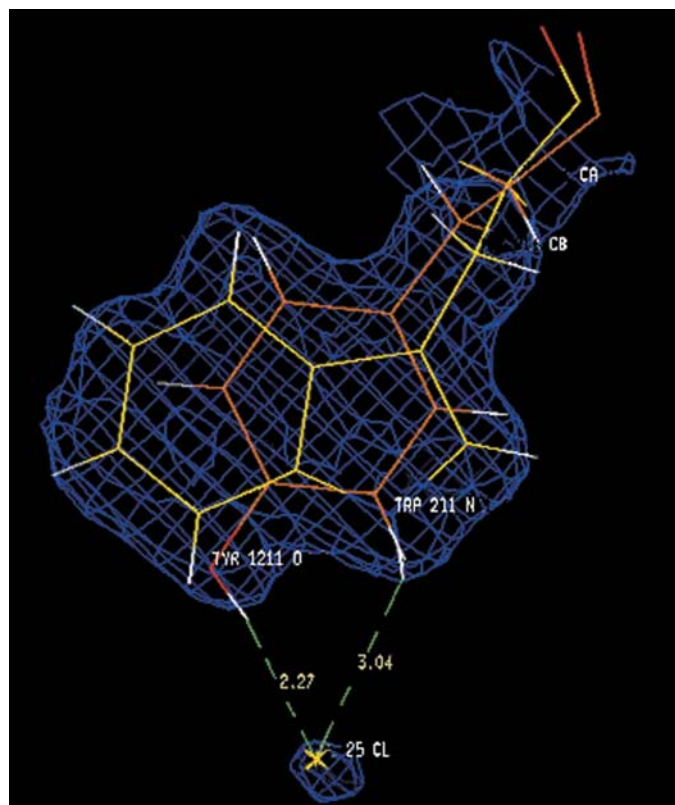


Figure 4

Trp/Tyr211 residue hydrogen bonded to Cl25 with superimposed $2F_o - F_c$ map at the 1σ level. This figure was produced using *Xfit* (McRee, 1999).

3.3. Hydrogen bonding of Trp (Tyr) side chains with chlorides and waters

Trp side chains form numerous hydrogen bonds with chlorine anions and H₂O molecules in the interchannel space in the Rb-gD complex (Table 4). No hydrogen-bond acceptors were found for Trp109, Trp111 and the less populated conformers of all five tryptophans displaying positional disorder (*i.e.* Trp309b, 213b, 313b, 411b and 413b). The Cl ions were found in 12 locations ranging in occupancy from 0.05 (1) to 0.36 (1) with B_{eq} values of 10–64 \AA^2 (Table 2). Although the occupancy of Cl25 of 0.05 (1) corresponding to about one electron only may not seem real, we are certain of the presence of a Cl ion owing to its participation as an acceptor in a hydrogen bond with both Trp211 and its replacement Tyr211 (Fig. 4) in the minor component gC. The total Cl⁻ occupancy in the asymmetric unit of 2.6 is lower than the total Rb⁺ occupancy of 2.8. The lack of charge balance is an indication of the degree of uncertainty in occupancy and B_{eq} values. Only one ethanol molecule was identified in the electron-density maps. Molecules of water are distributed among ten sites with occupancies between 0.09 and 0.98 and B_{eq} values of 3.9–82 \AA^2 . Of the ten partial waters in the interchannel space, only three are hydrogen bonded with Trp residues, while almost every one of the 12 chlorine anions makes at least one such bond and the average number of bonds is 2 (Table 5). Although Cl⁻ anions typically accept three hydrogen bonds (Allen, 2002), this requirement is hard to fulfil in the gramicidin crystal owing to geometrical restrictions (Fig. 1) and the

Table 5

Geometry of Rb⁺...carbonyl contacts for Rb⁺...O(C or N) distances less than 3.77 Å (in bold).

In the case of Rb⁺...O=C angles below 90° a contact was included only when the respective Rb⁺...C=O angle was also below 90° (indicated by an asterisk). Distances are given in angstroms and angles in degrees.

	Rb ⁺ ...O	Rb ⁺ ... O=C	Rb ⁺ ...C	Rb ⁺ ... C=O	Rb ⁺ ...N	Rb ⁺ ... N-C
Channel I						
Rb1						
202	3.33	102	3.80	59	4.05	116
114	3.69	102	4.12	61	4.80	131
201	3.77	128	4.63	40	5.12	
Rb2						
202	3.13	141	4.17	28	—	—
112	3.37	114	4.04	50	4.77	135
204*	3.61	84	3.69	77	3.70	96
Rb3						
206	3.46	100	3.88	61	4.70	—
108	3.48	136	4.43	33	5.20	—
110	3.56	90	3.77	71	3.89	111
208*	3.64	78	3.59	83	3.20	95
Rb4						
208	3.58	107	4.11	56	4.52	119
108	3.59	101	4.00	62	4.42	109
211a	3.67	98	4.03	64	3.81	80
Rb5						
215	3.17	116	3.85	48	3.78	78
212	3.38	111	4.00	52	4.66	122
107	3.42	113	4.05	51	3.80	93
105*	3.54	87	3.67	75	4.55	62
104	3.59	106	4.10	58	4.45	109
Rb6						
212	3.05	131	3.99	35	4.83	133
104	3.35	92	3.58	69	3.51	99
105	3.42	98	3.78	63	4.14	56
102	3.48	120	4.23	46	5.20	—
215	3.62	101	4.02	62	4.14	69
Rb7						
105	3.35	124	4.15	42	4.49	68
215*	3.44	79	3.42	81	4.10	71
214	3.47	98	3.86	63	4.36	122
102	3.48	100	3.89	62	4.36	122

lipophilic character of most side chains filling the space between the channels in the crystal. The Cl⁻ ions frequently make more than one hydrogen bond with ordered and disordered Trp residues, suggesting that anion type and size influences heterodimer crystallization.

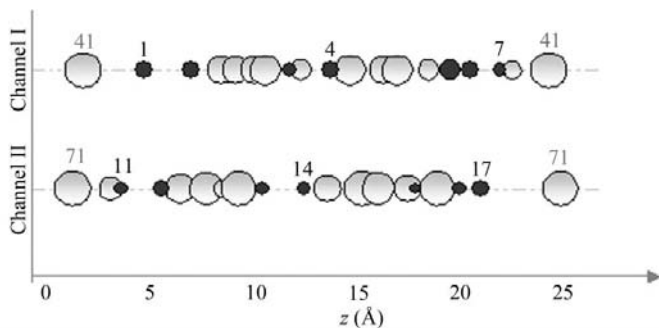


Figure 5

Distribution of Rb⁺ ions (black circles) and waters (gray circles) in gramicidin channels I and II in the crystal structure of the gD-RbCl complex from ethanol, shown along channel axis. The size of the circle is proportional to the occupancy factor. The origin is at the C1^α atom of strands 2 and 4.

Table 5 (continued)

	Rb ⁺ ...O	Rb ⁺ ... O=C	Rb ⁺ ...C	Rb ⁺ ... C=O	Rb ⁺ ...N	Rb ⁺ ... N-C
Channel II						
Rb11						
405	3.44	124	4.24	43	4.53	63
314	3.49	99	3.88	63	4.66	133
402	3.62	104	4.11	59	4.31	116
Rb12						
402	3.14	134	4.11	34	5.20	—
312	3.45	110	4.04	53	4.78	137
404*	3.46	87	3.61	73	3.54	97
405*	3.74	86	3.85	76	4.31	64
Rb13						
308	3.41	135	4.39	34	5.30	—
310	3.45	90	3.66	70	3.75	108
406	3.48	98	3.86	63	4.70	126
408*	3.67	75	3.55	85	3.15	94
Rb14						
308	3.62	100	4.03	62	4.52	113
408	3.64	103	4.11	60	4.54	118
411a	3.74	103	4.18	61	3.93	74
Rb15						
307	3.36	102	3.81	60	3.48	90
415	3.55	105	4.05	58	3.56	73
305*	3.72	81	3.73	80	4.72	87
Rb16						
302	3.16	117	3.86	47	4.60	129
305	3.30	107	3.86	55	3.88	81
414	3.37	94	3.67	66	3.82	111
412	3.73	129	4.61	39	5.50	—
Rb17						
305	3.20	130	4.12	36	4.27	94
414	3.26	107	3.82	55	4.61	126
302	3.70	103	4.13	61	4.32	113

Also of interest is the clustering of chlorine ions and waters. The largest cluster contains four Cl⁻ ions and one water molecule (with partial water occupancies totaling 1.35). The second largest cluster contains three Cl⁻ ions and one water molecule. There are also single anions with occupancies varying from 0.05 to 0.28. The average parameters of the hydrogen bonds are 2.5 Å and 144° for Cl⁻...H distances and N-H...Cl angles, respectively (see supplementary material¹).

3.4. Cations and water distributions in the double-stranded gramicidin channel

All rubidium positions in this structure were identified by the presence of anomalous scattering signals. Positions occupied by water only had no anomalous signal. In positions where rubidium and water share partial occupancy, it is difficult to obtain a reliable estimate of the percentage occupancy of each. In the Rb complex, up to seven Rb⁺ positions have been identified in each channel (Fig. 5). Their occupancies are between 0.11 and 0.35, their isotropic temperature factors range between 10.0 and 26.5 Å² and the average ion content is 1.59 and 1.22 in channels I and II, respectively (Table 4). The same number of sites (seven) was reported by Burkhart, Li *et al.* (1998) for the gD-CsCl complex.

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: DZ5031). Services for accessing these data are described at the back of the journal.

Ion-binding sites 4 and 14 (Figs. 5 and 6) are at the centers of the gramicidin channels. Cations in these sites are coordinated by amino acids related by non-crystallographic symmetry (Table 5): Rb4 is coordinated by Val108 (Rb \cdots O distance 3.59 Å), Val208 (3.58 Å) and Trp211a (3.67 Å) and Rb14 is coordinated by Val308 (3.62 Å), Val408 (3.64 Å) and Trp411a (3.74 Å). There are three binding sites on either side of the center of the channel. The distributions of ions in the three sites differ in occupancies and positions. Binding sites

are dispersed over a 7 Å range on one side, while on the other side the range is less than 3.2 Å (Fig. 5). The sum of the partial occupancies of the three sites is 0.61 on one side and 0.78 on the other side in channel I, while in channel II both sums are 0.53 despite very different occupancies in equivalent sites (Table 4). An extreme example of the difference between crystallographically independent channels is provided by the occupancies of sites 5 (channel I) and 15 (channel II), which vary from 0.11 to 0.35 (see supplementary material).

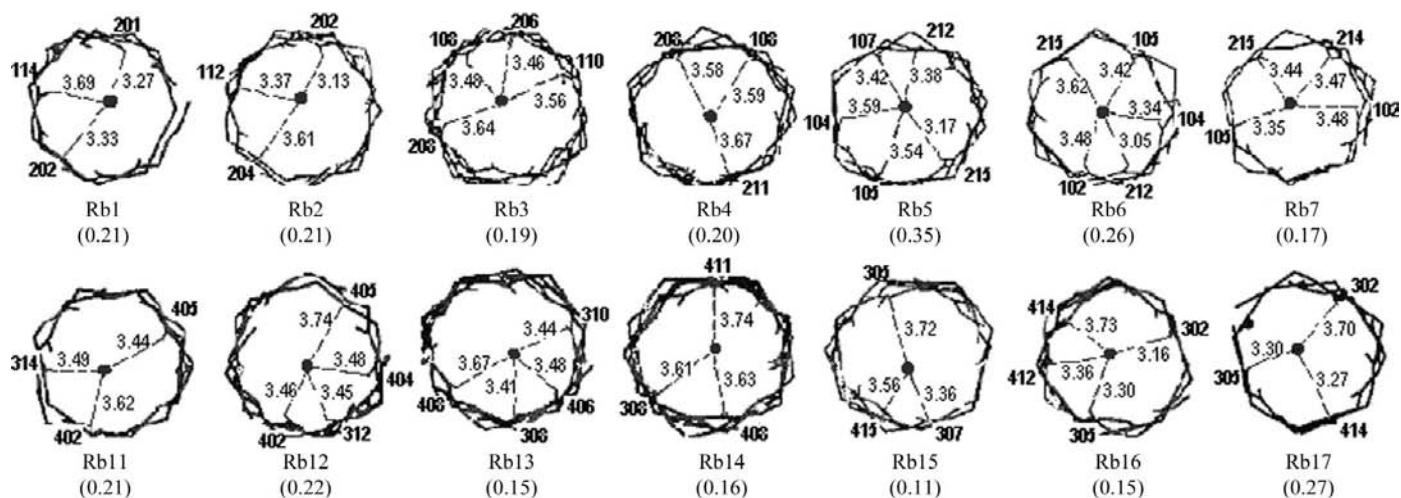


Figure 6 Rb \cdots O(carbonyl) short contacts (below 3.8 Å) viewed along [001] for channels I (top) and II (bottom) in the crystal structure of gD-RbCl.

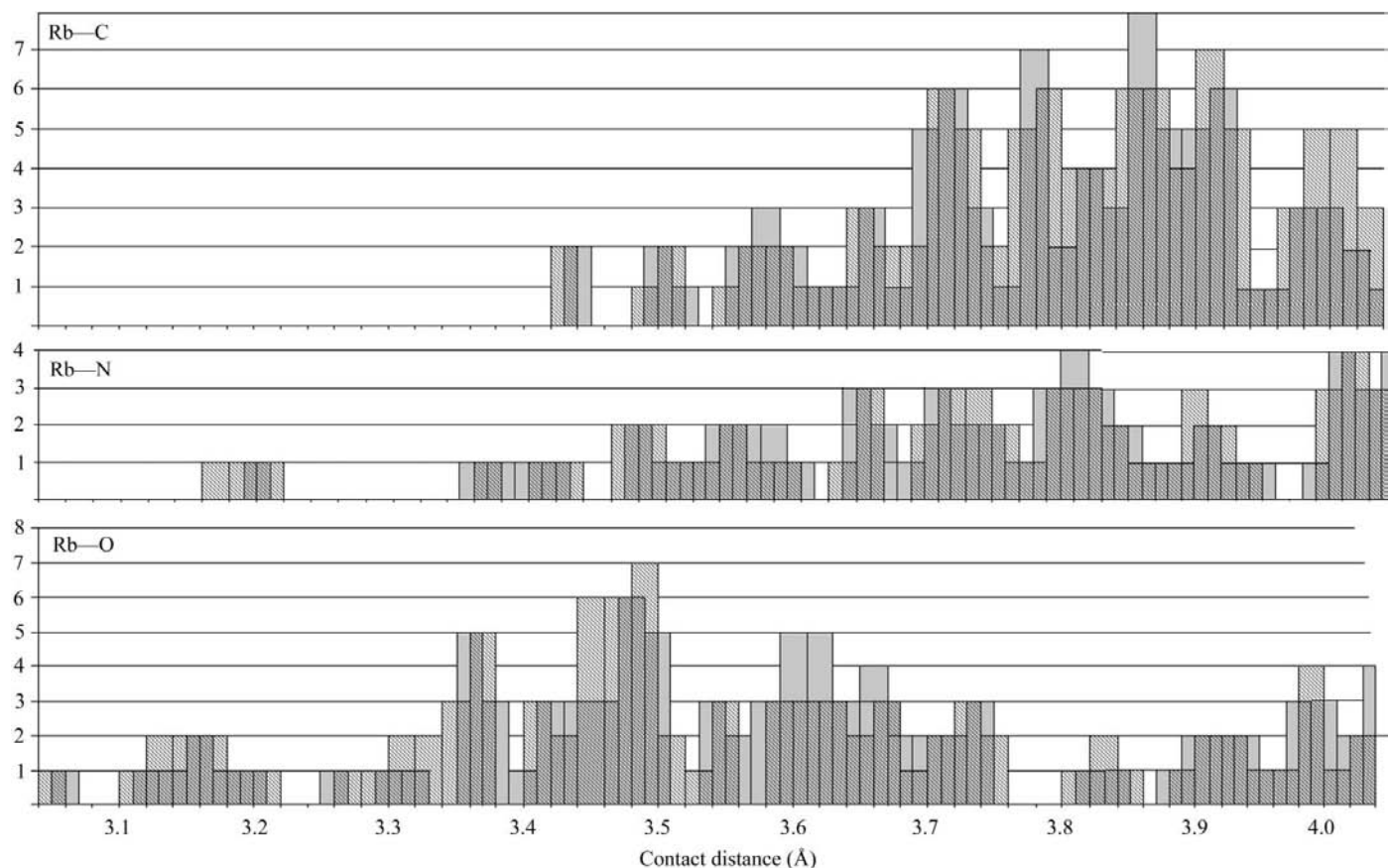


Figure 7 Distribution (histogram) of Rb \cdots O, Rb \cdots C and Rb \cdots N short contacts.

Similar asymmetry is also observed in the distribution of water molecules (Fig. 5), which occupy 11 sites in each channel. B_{eq} factors and occupancy factors for water molecules inside the channels are in the ranges 14.1–75.5 Å² and 0.22–1.0, respectively (Table 4). The average values are 18.7 and 22.7 Å² and 0.51 and 0.65 for channels I and II, respectively. Exceptions are the water molecules in the gaps between gramicidin channels in the crystal, which have extremely large temperature factors (Table 4). The asymmetry of rubidium ions and waters distribution correlates with the asymmetry of gramicidin double-stranded dimers, which are partial gA–gC heterodimers (see §3.1).

The total occupancy of waters within the channels is 5.6 and 7.1 in channels I and II, respectively, giving water:cation ratios of 3.5 and 5.8. The values are significantly smaller than those reported from microelectrochemical measurements, which showed seven waters crossing the channel for every Na⁺ cation (Tripathi & Hladky, 1998). The same number of waters in a channel was reported by Burkhardt *et al.* (1999).

Because more than one Rb⁺ is found per channel (Table 4), some channels must hold two cations at the same time and some may be filled with water only. Experimental evidence from Andersen & Procopio (1980) and Wolf & Roux (1997) suggest that cations reside at the opposite mouths of the channel, some 20 Å apart. In this structure, the distances between the two furthest rubidium ions are 18.0 and 17.9 Å in channels II and I, respectively. The estimated Rb⁺···(H₂O)_{*n*}···Rb⁺ distance depends on H₂O···OH₂ separations. If an average water-to-water distance were 2.4 Å and Rb⁺···OH₂ distance were 3.0 Å, the calculated Rb⁺···Rb⁺ distance would be 15.6, 18.0 and 20.4 Å for *n* = 5, 6 and 7 water molecules separating cations, respectively. However, if water-to-water distances were as large as 3.0 Å, separation of the furthest Rb cations would be 18, 21 and 24 Å, respectively. Thus, the value of 18 Å observed in this study would correspond to five or six waters between cations travelling through the channel.

3.5. Channel binding sites

One might expect ‘equatorial’ coordination contacts between cations in the channel and carbonyl O atoms in the ‘walls’ of the channel. However, the carbonyl groups of the peptide bonds are involved in antiparallel β-sheet hydrogen bonding that forms the channel wall. These carbonyl groups cannot deviate significantly from the wall and make Rb⁺···carbonyl group contacts through their *sp*² lone-pair orbitals typical of metal carbonyl complexes (Allen, 2002). Although carbonyl groups are distributed along the entire length of the channel, their positions on the walls make some sites better suited than others to cation localization. Theoretically, the average Rb···O(carbonyl) distance could be as large as 4 Å and the Rb···O=C angle as small as 90°. In fact, the distribution of Rb···O distances found in the current gramicidin structure (Fig. 7) shows that there are three types of contacts. The first type consists of distances below 3.2 Å with *M*⁺···O=C angles ranging from 116 to 141° (average

125°; Table 5). The second type includes distances between 3.30 and 3.75 Å with significantly smaller Rb⁺···O=C angles (Table 5). Often the angles are even smaller than 90° and sometimes the Rb⁺···C=O angles are also below 90° and the Rb ion lies between the C and O atoms of the carbonyl bonds. In some cases, the Rb⁺···N (amide) contacts are also very short (Table 5). These and earlier observations (Burkhardt *et al.*, 1999; Duax *et al.*, 2002, 2003) indicate that the cations are mainly coordinated by the π-electron clouds of carbonyl groups and entire peptide groups and not by the lone-pair electrons of the carbonyl oxygen. The coordination spheres of the rubidium cations are completed by two ‘axial’ water molecules.

Financial support from the Polish Committee for Scientific Researches under the project 3 T09A 047 26 (2004–2006) and Institute of General and Ecological Chemistry (PB-DS 47) is gratefully acknowledged. The use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Energy Research under Contract No. W-31-102-Eng-38.

References

- Allen, F. H. (2002). *Acta Cryst.* **B58**, 380–388.
 Andersen, O. S. & Procopio, J. (1980). *Acta Physiol. Scand. (Suppl.)*, **481**, 27–35.
 Arseniev, A. S., Barsukov, I. L. & Bystrov, V. F. (1985). *FEBS Lett.* **180**, 33–39.
 Becker, M. D., Greathouse, D. V., Koeppel, R. E. II & Andersen, O. S. (1991). *Biochemistry*, **30**, 8830–8839.
 Burkhardt, B. M., Gassman, R. M., Langs, D. A., Pangborn, W. A. & Duax, W. L. (1998). *Biophys. J.* **75**, 2135–2146.
 Burkhardt, B. M., Gassman, R. M., Langs, D. A., Pangborn, W. A., Duax, W. L. & Pletnev, V. (1999). *Biopolymers*, **51**, 129–144.
 Burkhardt, B. M., Li, N., Langs, D. A., Pangborn, W. A. & Duax, W. L. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 12950–12955.
 Bystrov, V. F. & Arseniev, A. S. (1988). *Tetrahedron*, **44**, 925–940.
 Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
 Doyle, D. A. & Wallace, B. A. (1997). *J. Mol. Biol.* **266**, 963–977.
 Duax, W. L., Burkhardt, B. M., Pangborn, W. A., Pletnev, V., Wawrzak, Z. & Glowka, M. (2002). *Adv. Supramol. Chem.* **8**, 245–282.
 Duax, W. L., Pletnev, V. & Burkhardt, B. M. (2003). *J. Mol. Struct.* **647**, 97–111.
 Dubos, R. J. (1939). *J. Exp. Med.* **70**, 1–10.
 Dubos, R. J. & Hotchkiss, R. H. (1941). *J. Biol. Chem.* **141**, 155–162.
 Engh, R. A. & Huber, R. (1991). *Acta Cryst.* **A47**, 392–400.
 Gross, E. & Witkop, B. (1965). *Biochemistry*, **4**, 2495–2501.
 Hotchkiss, R. D. (1944). *Adv. Enzymol.* **4**, 153–199.
 Hu, W. & Cross, T. A. (1995). *Biochemistry*, **34**, 14147–14155.
 Killian, J. A., Prasad, K. U., Hains, D. & Urry, D. W. (1988). *Biochemistry*, **27**, 4848–4855.
 Langs, D. A. (1988). *Science*, **241**, 188–191.
 Langs, D. A., Smith, G. D., Courseille, C., Précigoux, G. & Hospital, M. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 5345–5349.
 Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
 Lomize, A. L., Orekhov, V. & Arseniev, A. S. (1992). *Bioorg. Khim.* **18**, 182–200.
 McRee, D. E. (1999). *J. Struct. Biol.* **125**, 156–165.
 Merritt, E. A. & Murphy, M. E. P. (1994). *Acta Cryst.* **D50**, 869–873.
 Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

- Pascal, S. M. & Cross, T. A. (1993). *J. Biomol. NMR*, **3**, 495–513.
- Pressman, B. C. (1965). *Proc. Natl Acad. Sci. USA*, **53**, 1076–1083.
- Ramachandran, G. N. & Saisekharan, V. (1968). *Adv. Protein Chem.* **23**, 283–438.
- Sarges, R. & Witkop, B. (1965). *J. Am. Chem. Soc.* **87**, 2011–2020.
- Sheldrick, G. M. & Schneider, T. R. (1997). *Methods Enzymol.* **277**, 319–343.
- Tripathi, S. & Hladky, S. B. (1998). *Biophys. J.* **74**, 2912–2917.
- Urry, D. W., Goodall, M. C., Glickson, J. D. & Mayers, D. F. (1971). *Proc. Natl Acad. Sci. USA*, **68**, 672–676.
- Veatch, W. R., Fossel, E. T. & Blout, E. R. (1974). *Biochemistry*, **13**, 5249–5256.
- Wallace, B. A. (1998). *J. Struct. Biol.* **121**, 123–141.
- Wallace, B. A. & Ravikumar, K. (1988). *Science*, **241**, 182–187.
- Weinstein, S., Wallace, B. A., Morrow, J. S. & Veatch, W. R. (1980). *J. Mol. Biol.* **143**, 1–19.
- Wolf, T. B. & Roux, B. (1997). *Biophys. J.* **72**, 1930–1945.
- Zhang, Z., Pascal, S. M. & Cross, T. A. (1992). *Biochemistry*, **31**, 8822–8828.