5-Å FOURIER MAP OF GRAMICIDIN A PHASED BY DEUTERIUM-HYDROGEN SOLVENT DIFFERENCE NEUTRON DIFFRACTION

ROGER E. KOEPPE II
Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701

BENNO P. SCHOENBORN
Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT Crystals of ion-free gramicidin A ($P2_12_12_1$: $a = 24.61$, $b = 32.28$, $c = 32.52$) have been investigated using neutron diffraction. A difference analysis of crystals soaked in ethanol/H_2O as opposed to ethanol-d_6/D_2O has led to single isomorphous replacement Fourier projections of the structure at 5-Å resolution. The gramicidin dimer appears to be a 32-Å-long cylinder oriented parallel to the *c*-axis in these crystals.

Gramicidin A from *Bacillus brevis* is probably the best-characterized transmembrane cation channel (Weinstein et al., 1980; Finkelstein and Andersen, 1981; Urry et al., 1982). This pentadecapeptide has been crystallized from methanol and ethanol (Cowen and Hodgkin, 1953; Veatch, 1973) and in complexes with CsSCN and KSCN (Koeppe et al., 1978; 1979), but the phase problem has not been solved either by direct methods or by isomorphous replacement. The conformation of gramicidin A is dramatically different in the ion-free and ion-bound crystal forms (Koeppe et al., 1978; Wallace et al., 1981). The most stable ion sites found in the crystal (Koeppe et al., 1979) agree with those found in solution and in membranes (Andersen et al., 1981; Urry et al., 1982; Hinton et al., 1981), and the dimensions of the gramicidin A molecule in the ion-bound crystals are consistent with those of the $\beta^{6,3}_{3,3}$-helix (Koeppe and Kimura, 1983), a structure first proposed by Urry (1971) and now generally agreed to be an accurate representation of the active channel in membranes (Urry et al., 1975; Bamberg et al., 1977; Weinstein, et al., 1980).

The ion-free form of gramicidin A in either methanol or ethanol has a structure whose identity and relation to the membrane conformation are currently unknown. The crystal structure appears to be the same whether the gramicidin A is crystallized from methanol or from ethanol (Koeppe et al., 1978). The circular dichroism (CD) spectrum of gramicidin A in these alcohol solvents is concentration dependent, and indicates that the predominant conformer at high concentration is quite different from the membrane conformation (Urry et al., 1975; Wallace et al., 1981). Nevertheless, the ion-free crystal structure of gramicidin A is of interest for the following reasons: (*a*) it would be useful to know the full range of folding patterns available to the peptide and to understand interconversions among various conformers, (*b*) an alteration in the folding pattern may be involved when the peptide inserts into a membrane, (*c*) conformations other than the membrane channel may be important for other biochemical functions of gramicidin A such as the regulation of RNA polymerase (Sarkar et al., 1977) by interference with binding to DNA (Sarkar et al., 1978), and, finally, (*d*) the form that appears in the crystal could possibly be one of the minor conformers present in the mother liquor (for example, Z-DNA crystallizes under conditions where B-DNA is the predominant species in solution [Crawford et al., 1980]), so that the crystalline conformation of gramicidin A need not necessarily represent the conformation that dominates the CD spectrum of the solution from which the crystals were grown.

Because heavy atoms will not diffuse into crystals of gramicidin A (Veatch, 1973), and co-crystallization of gramicidin A with heavy atoms produces crystals that are not isomorphorous with the ion-free crystals (Koeppe et al., 1978), we have turned to hydrogen-deuterium difference neutron diffraction (Schoenborn, 1976) as a potential source of phase information for crystals of ion-free gramicidin A. This paper reports an analysis of the changes in the diffraction pattern due to the substitution of deuterium for hydrogen in the solvent of crystals of gramicidin A. We find that the data are amenable to treatment analogous to the treatment of data for a multisite heavy atom derivative in x-ray diffraction, and that projection Fourier maps of the structure can be calculated by using the centric reflections, phased on the best eight deuterium substitution sites. These results suggest that the method may be extended to

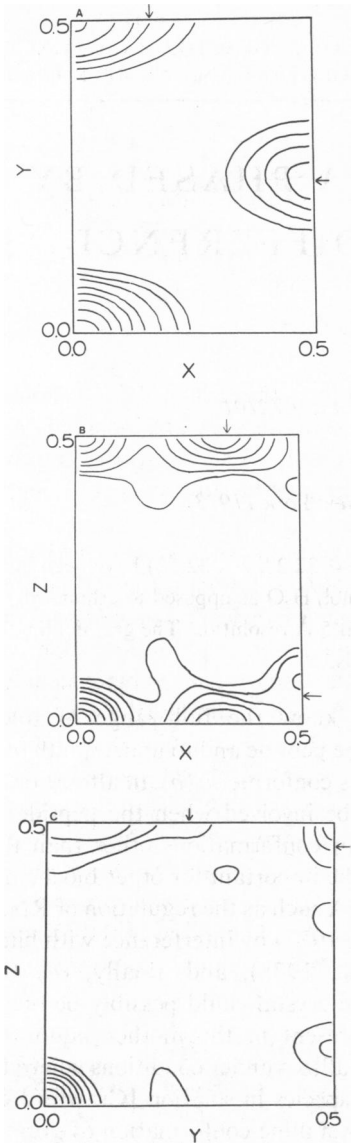

FIGURE 1 Difference neutron Patterson map calculated from the deuterated solvent and hydrogenated solvent data sets for gramicidin A at 5-Å resolution with $(|F_D| - |F_H|)^2$ used as coefficients. (*A*) HKO projection. (*B*) HOL projection. (*C*) OKL projection.

yield three-dimensional phases by including a third data set recorded from a crystal containing partially deuterated solvent.

Crystals of HPLC (high-performance liquid chromatography)-purified valine gramicidin A (Koeppe and Weiss, 1981) were grown from ethanol by our previously used procedure (Koeppe et al., 1978). The use of HPLC-purified material resulted in larger single crystals for neutron diffraction (up to 20 mm^3) than had previously been available. Individual crystals were removed from the mother liquor and stored in ethanol/H_2O 1:1, a peptide-free liquid in which the crystals are stable with no change in unit-cell dimensions (Koeppe et al., 1978). One of the crystals was soaked prior to data collection in a completely deuterated solvent (ethanol-d_6/D_2O 1:1) for 1 mo, with four changes of solvent. These crystals are orthorhombic, space group $P2_12_12_1$, with cell parameters a = 24.61, b = 32.28, c = 32.52. Each crystal was sealed in a quartz capillary containing a small amount of the appropriate ethanol-water solution, and mounted on a four-circle diffractometer with the crystallographic c^* axis parallel to the instrument ϕ axis.

The neutron beam from the Brookhaven High-Flux Beam Reactor was focused and monochromatized (1.55 Å) by means of a bent pyrolytic graphite monochromator, and then passed through a $\lambda/2$ filter. Diffraction data were collected with normal beam rotation geometry, as described by Cain et al. (1975), except that a two-dimensional position-sensitive detector (Alberi, 1975) was used instead of a series of linear detectors. The data were recorded as three-dimensional arrays of counts in X and Y detector channels as the crystal was rotated about the ϕ axis through 19 steps of 0.06° surrounding each precalculated reflection position. The (X, Y, ϕ) arrays were reduced to two-dimensional arrays by summing along Y, and then the peaks were integrated by the automated ellipse-fitting procedure of Spencer and Kossiakoff (1980).

The reduced intensity data were scaled in two steps. In step one, each data set was scaled so that the Patterson origin agreed with $\Sigma_i n_i f_i^2$ for a unit cell consisting of eight gramicidin monomers of composition $C_{99}H_{140}N_{20}O_{17}$ (21 of the H exchangeable in deuterated solvent), and having ~10 water molecules and 4 ethanol molecules per gramicidin monomer (based on 15% solvent in the crystal [Veatch, 1973], and equal weights of ethanol and water). This method takes into account the increase in total scattering length density that accompanies the substitution of deuterium for hydrogen, and assumes that a large fraction of the solvent is well ordered (a good assumption for gramicidin with only 15% solvent). The second step in scaling involved a small correction K_D for the deuterium data, derived by using only centric reflections in the expression (Blundell and Johnson, 1976)

$$K_D = (\sum_{\text{centrics}} F_D F_H)/(\sum_{\text{centrics}} F_H^2).$$

Because this paper deals mainly with the centric data (acentric data used only for Fig. 3), we were most concerned with correct scaling of the centric reflections. After scaling, the R factor on $\sqrt{I}$ between the H- and D-data sets was 28%, in agreement with the predicted value upon substitution of ~65 H's for D's per monomer of gramicidin. Inclusion of a sin θ dependence of the scale factor had little effect (lowered the R factor to 27%), thus supporting the expectation that deuterium substitution would have little effect on the temperature factor of a crystal.

The scaled data were used to calculate three centrosymmetric Patterson projections, with $(|F_D| - |F_H|)^2$ used as coefficients (Fig. 1). The HKO projection had peaks at x =

TABLE I
D-H SUBSTITUTION SITES IN FRACTIONAL COORDINATES

Relative occupancy	x	y	z
4.0	0.06	0.15	0.98
4.0	0.38	0.42	0.16
1.2	0.14	0.44	0.50
1.7	0.24	0.16	0.05
2.4	0.16	0.52	0.19
0.5	0.06	0.36	0.48
0.3	0.34	0.32	0.22
1.0	0.17	0.17	0.46

0, $y = 1/2$ and $x = 1/2$, $y = 1/4$, reminiscent of the x-ray self Patterson map for this crystal form of gramicidin A (Koeppe et al., 1978) and suggesting significant deuterium occupancy at the centers of channels (near $x = 0$, $y = 1/8$, and symmetry-related sites). By analogy with the x-ray Patterson, one might have expected the strongest deuterium substitution to be almost continuous along the length of a channel at all z-levels along the line $x = 0$, $y = 1/8$. However, the HOL and OKL difference Patterson projections show instead a marked localization of sites along the z-axis, and suggest that there may be significant sites at (x, y) values other than those representing the centers of channels. In fact, all three projections have strong Harker peaks consistent with the site (0.08, 0.12, ± 0.02) (see arrows in Fig. 1).

A first impression is that the difference Patterson projections look remarkably simple, given the high level of deuterium substitution that would be expected. Upon further reflection, we reasoned that disordered solvent would contribute little to the difference maps, and that at low resolution various deuteriums on the same solvent molecule would appear as only one site. Both these considerations would tend to reduce the number of expected sites. Therefore, it seemed plausible that the difference between the two data sets could be approximated by a number of discrete sites of deuterium substitution, as in the case of a multisite heavy atom derivative in x-ray diffraction. To test this approach, a single site at (0.00, 0.125, 0.00) was subjected to least-squares phase refinement in three centrosymmetric projections (Dickerson et al., 1968). The site moved during the refinement to the Patterson-predicted position of (0.08, 0.12, −0.02), and refined to a centric R factor of 0.46 at 5-Å resolution (Table I). This site was used to calculate $(F_{\text{gram D}} - F_{\text{gram H}} - f_{\text{site}})\,\phi_{\text{site}}$ difference Fourier projections, which showed two possible additional sites. These new sites were refined individually and then in pairs with the first site. A difference Fourier map based on three sites showed eight potential additional weak sites, only two of which would refine satisfactorily. Finally, three peaks from a five-site difference Fourier were accepted on the basis of individual refinement and pairwise refinement with the first site. Table II lists the coordinates of the eight

TABLE II
CENTRIC ΔF R-FACTOR AS A FUNCTION OF RESOLUTION AND NUMBER OF SITES

Number of sites	8 Å	5 Å	4 Å	3 Å	2.5 Å
1	0.386	0.457	0.487		
2		0.372			
4		0.300	0.343	0.405	
5				0.362	
8				0.281	0.332

sites refined all together, while Table I shows the change in the R factor as more sites are included. Additional sites beyond eight produced no significant improvement in the refinement, perhaps because only 477 centric reflections were available.

Although the eight sites shown in Table II may not represent a unique solution to the H-D difference diffraction data, they nevertheless provide one reasonable solution that fits the data very well and, as such, can be used to generate single isomorphous replacement (SIR) phases. Fig. 2 shows the HKO and HOL projection Fourier maps phased by single isomorphous deuterium replacement. These maps were calculated by using figure of merit weighting (mean $m = 0.72$ for 5-Å data). The HKO projection resembles flattened, distorted cylinders, in a packing arrangement similar to that deduced from x-ray data (Koeppe et al., 1978). The asymmetry of the cylindrical projections is probably due to the distribution of large side chains (primarily indole rings) around the perimeter

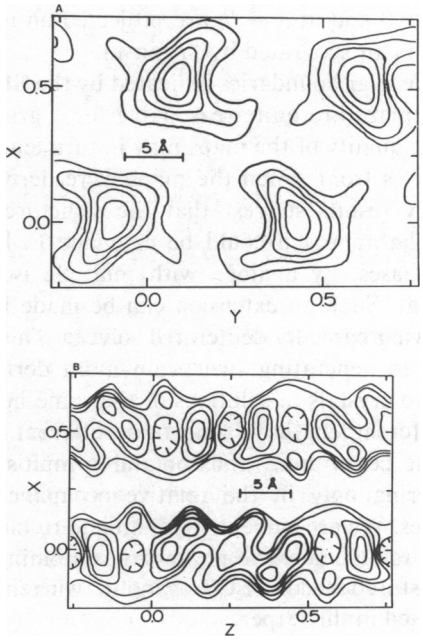

FIGURE 2 Fourier maps of gramicidin A at 5-Å resolution computed from single isomorphous replacement phases based on the eight deuterium-hydrogen solvent difference sites of Table I. (*A*) HKO projection. (*B*) HOL projection.

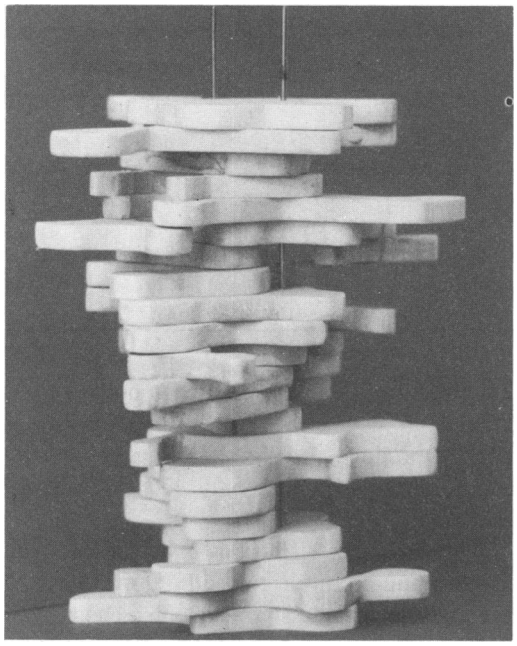

FIGURE 3 A photograph of a wooden model made by assembling sections of a 5-Å Fourier map of gramicidin A computed from single isomorphous replacement phases based on the eight deuterium-hydrogen solvent difference sites of Table I.

of a helix formed by the gramicidin A backbone. Each of the distorted cylinders in the HKO projection represents one asymmetric unit, which consists of a dimer of gramicidin A. The HOL projection illustrates a side view of the cylinders in the unit cell. The molecular boundaries in the HOL projection are particularly clear since pairs of cylinders at $x = 0$ and at $x = \frac{1}{2}$ are projected on top of each other (each pair separated by $1/2$ in y).

The molecular boundaries indicated by the SIR neutron projection maps are quite reasonable for a gramicidin A dimer. The quality of the maps may in turn lend credence to the phases from which the maps were derived. These preliminary results suggest that the structure could be solved if the approach could be extended to handle the acentric phases, by analogy with multiple isomorphous replacement. Such an extension can be made by using a crystal having partially deuterated solvent. This would be analogous to generating two heavy-atom derivatives by soaking two crystals in solutions of the same heavy atom, but for different lengths of time, or at different concentrations of the heavy atom, thus obtaining multisite derivatives differing only in the relative occupancies of the various sites. We are currently planning to resolve the SIR ambiguity for the acentric phases by combining the data from a crystal soaked in H_2O/ethanol-d_5 with the two data sets discussed in this paper.

Fig. 3 shows a balsa wood model constructed by gluing together sections from the three-dimensional SIR Fourier map at 5-Å resolution. Map pieces corresponding to one asymmetric unit, or a dimer of gramicidin A, were included in the model. The key to aligning any particular helical structure to fit such a low-resolution map will be to locate the positions of the eight tryptophan side chains. Although the wooden model has numerous peripheral features that could resemble indole rings, a unique alignment of the amino acid sequence with the SIR map is not possible. An improved model should be available when additional phase information from crystals soaked in partially deuterated solvent is included. At that time, we expect to be able to trace the amino acid sequence through the Fourier map.

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