Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12828–12831, December 1994
Biochemistry

Subunit stoichiometry of staphylococcal α-hemolysin in crystals and on membranes: A heptameric transmembrane pore

J. ERIC GOUAUX*†, ORIT BRAHA‡, MICHAEL R. HOBAUGH*§, LANGZHOU SONG*§, STEPHEN CHELEY‡, CHRISTOPHER SHUSTAK‡, AND HAGAN BAYLEY†‡

*Department of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th Street, Chicago, IL 60637; and ‡Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545

Communicated by William N. Lipscomb, September 15, 1994

ABSTRACT **Elucidation of the accurate subunit stoichiometry of oligomeric membrane proteins is fraught with complexities. The interpretations of chemical cross-linking, analytical ultracentrifugation, gel filtration, and low-resolution electron microscopy studies are often ambiguous. Staphylococcal α-hemolysin (αHL), a homooligomeric toxin that forms channels in cell membranes, was believed to possess six subunits arranged around a sixfold axis of symmetry. Here, we report that analysis of x-ray diffraction data and chemical modification experiments indicate that the αHL oligomer is a heptamer. Self-rotation functions calculated using x-ray diffraction data from single crystals of αHL oligomers show a sevenfold axis of rotational symmetry. The αHL pore formed on rabbit erythrocyte membranes was determined to be a heptamer by electrophoretic separation of αHL heteromers formed from subunits with the charge of wild-type αHL and subunits with additional negative charge generated by targeted chemical modification of a single-cysteine mutant. These data establish the heptameric oligomerization state of the αHL transmembrane pore both in three-dimensional crystals and on a biological membrane.**

As exemplified by studies on cholera toxin (1), chemical cross-linking and electron microscopy may not provide precise and unequivocal information on the subunit stoichiometry of oligomeric proteins. Complete cross-linking of an oligomeric protein can give both cyclic and linear species, which may migrate differently on denaturing polyacrylamide gels, giving the false indication of an additional subunit (1). Dynamic or static rotational disorder about the axis of molecular symmetry in two-dimensional crystals can yield misleading diffraction data (1). Resolution of the subunit composition of cholera toxin was achieved by the collection and analysis of high-resolution x-ray diffraction data (2). A second example is aerolysin, a pore-forming protein secreted by *Aeromonas hydrophila*. Aerolysin was thought to form a pentamer or a hexamer (3) until a recent examination by rotational correlation analysis of particles in averaged electron micrographs suggested that it is a heptamer (4). However, this conclusion is being reevaluated (5). The gap junction connexon provides another example of an oligomeric membrane protein for which the number of subunits is still unclear. The connexon was believed to be a hexamer (6), but recent work suggests that it may be a pentamer (7).

Reservations concerning the reliability of methods employed in determining the subunit stoichiometry of other membrane proteins have led us to reexamine the quaternary structure of α-hemolysin (αHL), a protein that is a model system for studying membrane protein assembly (8–11), and that has potential applications in biotechnology [e.g., as a component of immunotoxins (12) or an element in biosensors (13)]. The αHL polypeptide of 293 amino acids is secreted by *Staphylococcus aureus* as a water-soluble monomer and assembles upon contact with lipid bilayers or the detergent deoxycholate (DOC) to form oligomeric cylindrical pores 1–2 nm in internal diameter (14). Mutagenesis experiments have revealed two assembly intermediates, a membrane-bound monomer and a nonlytic oligomer (10, 11). Both the water-soluble monomer and the fully assembled pore contain largely β-sheet secondary structure with little α-helical structure (9, 15). Conformational analysis by limited proteolysis suggests that monomeric αHL in solution contains two domains that are separated by a central glycine-rich loop. The loop becomes occluded upon assembly (9, 10), and a segment of it lines the lumen of the transmembrane channel of the pore (13, 16).

Estimates of five, six, or seven subunits for the stoichiometry of the αHL oligomer were originally obtained by direct examination of electron micrographs (17). More recently, application of rotational averaging techniques to views of single particles in membranes and image reconstruction from two-dimensional hexagonal crystals yielded a structure with six symmetrically arranged peaks (18, 19). Results from SDS/PAGE (αHL oligomers are stable in SDS-containing solutions) (8, 20), gel filtration (8, 20), sedimentation analysis (8, 20), light scattering (15), and chemical cross-linking (9) all supported the view that the αHL pore was a hexamer, whether it was formed on lipid bilayers or by the action of DOC. In contrast, image reconstruction from two-dimensional tetragonal arrays of αHL oligomers suggested that each oligomer contained four subunits, and it was proposed that this form of αHL represented a nonlytic oligomeric precursor of the pore (21, 22).

We have devised an alternative approach for the determination of subunit stoichiometry on biological membranes based on chemical modification and gel-shift electrophoresis. When the technique is applied to αHL, our results suggest that the pore is a heptamer. This conclusion has been independently derived by analysis of self-rotation functions calculated from single-crystal x-ray diffraction data.

MATERIALS AND METHODS

X-Ray Diffraction. Initial crystallization conditions were discovered using a systematic two-dimensional grid of ammonium sulfate and pH and αHL oligomer solubilized in 25 mM β-octyl glucoside (23–25). Microcrystals grew readily at concentrations of ammonium sulfate between 2.5 and 3.0 M

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: αHL, α-hemolysin; DOC, deoxycholate; IVTT, *in vitro* transcription and translation; IAM, iodoacetamide; IASD, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2-2′-disulfonate; rRBCM, rabbit erythrocyte membrane; MME, monomethyl ether; DTT, DL-dithiothreitol; WT-αHL, wild-type αHL.

†To whom reprint requests should be addressed.
§M.R.H. and L.S. made equal contributions to the crystallographic component of this work.

at pH 7.4 at room temperature using the hanging drop technique. With only ammonium sulfate as the precipitant, crystallization occurred either very near or under conditions of phase separation. Successively finer grids of ammonium sulfate concentration and pH did not improve the crystal size. In addition, the small crystals (largest dimension < 0.05 mm) generally grew in aggregates. Optimization of the crystallization conditions was achieved by (*i*) supplementing the protein solution with low concentrations of the monomethyl ether (MME; ref. 26) variants of PEG 550, 2000, and 5000, (*ii*) including sodium cacodylate at pH 6.0, and (*iii*) by raising the protein concentration to 7–9 mg/ml. Final conditions for the precipitant solution were 2.5 M ammonium sulfate, 0.25% PEG 5000 MME, and 0.075 M sodium cacodylate. These conditions decreased the formation of nuclei, prevented the growth of multiple crystals, and resulted in the production of crystals of sufficient size for high-resolution data collection.

For the x-ray diffraction experiments, a crystal was transferred from the growth solution to a siliconized glass capillary. An artificial mother liquor composed of ammonium sulfate, PEG 5000 MME, sodium cacodylate, and β-octyl glucoside was used to float the crystal into the capillary. Excess solvent was removed from the crystal, the crystal was flanked with small volumes of the artificial mother liquor, and the capillary was sealed with glue.

A native data set was collected to 3.5 Å resolution using an Enraf Nonius (Bohemia, NY) FAST area detector. Cu K_α x-rays were produced from an Elliott (Bohemia, NY) GX-21 rotating anode, operating at 40 kV and 70 mA with a 300-μm focus cup and a graphite monochromator. Frames of 0.1° in ϕ were taken using an exposure time of 2 min. The detector was placed at a distance of 100 mm from the crystal and was offset by 12° in 2Θ. Data were collected at room temperature. Under these conditions, the crystal decay after 6 days of continuous irradiation was <25%, based on comparing selected reflections on the same frame at the beginning and end of data collection. This relatively long lifetime enabled a complete data set to be collected from a single crystal.

Determination of the orientation of the crystal, refinement of the crystal and detector parameters, and integration of the measured intensities was performed using the programs MADNES (27) and PROCOR (28). Batches of data covering ranges in ϕ of 45–90° were scaled and merged using the Fox and Holmes algorithm (29) as implemented in the CCP4 computer program package (30).

Chemical Modification and Gel-Shift Electrophoresis. Radiolabeled αHL polypeptides were prepared by *in vitro* transcription and translation (IVTT) in the presence of [^{35}S]methionine (31). Rabbit erythrocyte membranes (rRBCMs) were prepared by hypotonic lysis of washed rabbit erythrocytes and suspended in 10 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.4) and 150 mM NaCl containing bovine serum albumin at 1 mg/ml.

To obtain αHL oligomers from premodified polypeptides, chemical modification of the single-cysteine mutant T292C (10) with 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2-2′-disulfonate (IASD; Molecular Probes) or iodoacetamide (IAM) was carried out directly on the IVTT mix. The mix (12 μl) was prereduced by the addition of 1.0 M sodium phosphate at pH 8.5 (9 μl) and 10 mM DL-dithiothreitol (DTT; 3 μl). After 5 min at room temperature, 100 mM IASD (6 μl in water) or 100 mM IAM (6 μl in water) was added, and the mixture was incubated for 2 h at room temperature, followed by the addition of 120 mM DTT (6 μl). rRBCMs (1.5 mg/ml, 10 μl) were then incubated with the αHL preparations that had been mixed in different ratios as indicated in the legend of Fig. 2 (10 μl total), for 60 min at 20°C. The membranes were recovered by centrifugation at 14,000 × *g* for 4 min at room temperature, dissolved in 1× electrophoresis loading buffer (32), and heated for 5 min in SDS solution at 45°C, a temperature at which αHL oligomers are stable.

αHL oligomers formed from underivatized αHL polypeptides were chemically modified after assembly on membranes. rRBCMs (10 μl) were treated with wild-type αHL (WT-αHL)/T292C mixtures (5 μl total, in IVTT mix) for 1 h at 20°C. After recovery by centrifugation, the membranes were resuspended in 0.43 M sodium phosphate at pH 8.5 (7 μl) and mixed with 10 mM DTT (1 μl). After 5 min at room temperature, 100 mM IASD (2 μl in water) was added. The mixture was incubated for 2 h at room temperature before the addition of 120 mM DTT (2 μl), followed by 2× loading buffer (12 μl). Samples were heated for 5 min at 45°C.

The αHL oligomers were subjected to separation by SDS/PAGE according to Laemmli (32) in 35-cm-long 5% gels at 4°C for 36 h at 160 V. Sodium thioglycolate (0.1 mM) was added to the upper (cathode) buffer to quench reactive species remaining after polymerization of the gel (33).

RESULTS AND DISCUSSION

Crystallographic Analysis. The αHL oligomer was obtained by incubating the monomer with DOC (8), followed by purification using gel-filtration chromatography. Crystallization of the oligomeric form of αHL solubilized in β-octyl glucoside was effected by equilibrating a solution containing equal volumes of protein and reservoir solution (ammonium sulfate/PEG 5000 MME/sodium cacodylate) against the reservoir solution. The protein used to grow the three-dimensional crystals of αHL had identical properties to the αHL oligomer reported in other studies (8, 20) based on SDS/PAGE, gel filtration, and analytical sedimentation equilibrium and velocity studies (M.R.H., L.S., and J.E.G., unpublished results). In addition, analysis of dissolved crystals by SDS/PAGE showed that the protein in the crystal was the same as the material used in the crystallization—i.e., oligomerization to a different subunit stoichiometry had not occurred during the crystallization process.

The space group for these crystals is *C*2. The unit cell dimensions are a = 151.9 Å, b = 136.8 Å, c = 135.1 Å, and β = 91.4°, and the crystals diffract to 1.8 Å resolution. From a single crystal approximately 0.2 × 0.2 × 0.5 mm in size, a total of 86,847 observations of 33,863 independent reflections were collected. These data were scaled, merged, and reduced, giving an R_{merge} of 0.048 for data to 3.5 Å resolution, where the average $I/\sigma(I)$ was 13.5.

On the basis of the volume of the asymmetric unit and the mass of an αHL heptamer, the volume to mass ratio, or V_M value (34), is 3.0 Å^3/Da for a single oligomer per asymmetric unit. In the cases of other membrane protein crystals, V_M values range from 5.1 Å^3/Da for the *Rhodopseudomonas viridis* photosynthetic reaction center (35) to 3.5 Å^3/Da for the ScrY porin from *Escherichia coli* (36). For water-soluble proteins, V_M values are generally smaller, with values ranging from 4.4 Å^3/Da to 1.8 Å^3/Da; the most common values are near 2.2 Å^3/Da, and the median value is 2.6 Å^3/Da (34). Two pentameric, hexameric, or heptameric oligomers per asymmetric unit would give an unreasonably densely packed crystal. Although it is possible for one-half of a hexameric oligomer to reside in the asymmetric unit, this would give a V_M of 7.0 Å^3/Da, a value that is not consistent with the mechanical robustness and excellent diffraction characteristics of this crystal form. Consequently, the asymmetric unit most probably contains a single oligomeric entity.

Self-rotation functions (37) were calculated by using the x-ray diffraction data described above and the program POLARRFN (30). Resolution ranges between 3.5 and 20 Å, 4.0 and 10 Å, 5.0 and 10 Å, and 6.0 and 20 Å and Patterson space integration radii of 20, 25, 30, and 35 Å were explored. The rotation angle κ was sampled every 3°, and the spherical polar

angles ω and ϕ were sampled every 5°. The self-rotation function, $R(\kappa, \phi, \omega)$, contains strong peaks for κ values of 51°, 102°, and 154° and ϕ and ω values of 45° and 90°, respectively (Fig. 1). For the self-rotation function calculated using data between 3.5 and 20 Å and an integration radius of 20 Å, the minimum density in the map is −18.4, the maximum is 100.0, the mean is 10.0, and the rms deviation from the mean is 22.8. Therefore, the peaks for the sevenfold axis of rotational symmetry are greater than the mean density by more than 3 times the rms deviation from the mean. Strong peaks defining a sevenfold axis of rotational or screw-axis symmetry were also found using the other resolution ranges and integration radii listed above, although the signal-to-noise ratios were not as large.

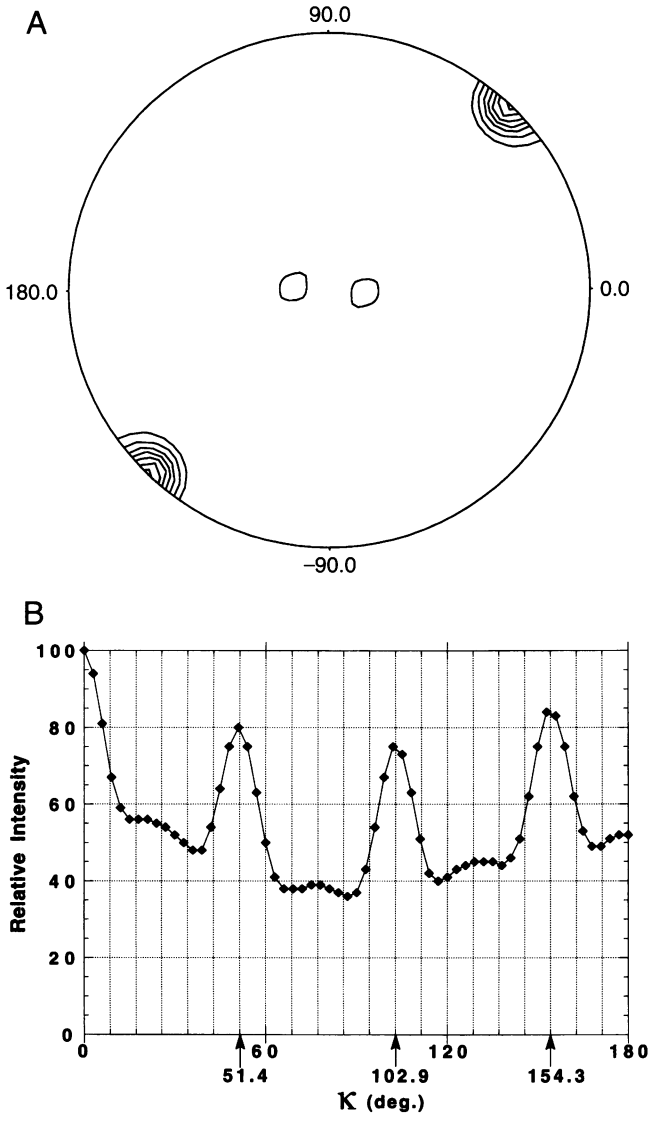

FIG. 1. αHL oligomers formed in DOC display sevenfold rotational symmetry in three-dimensional crystals. (*A*) The κ = 102° section of the self-rotation function, described in terms of the rotation angle κ and the spherical polar coordinates ω and ϕ. ω is defined as the rotation from the pole, and ϕ is the angle around the equator, on the plane of the page. The crystallographic twofold axis is perpendicular to the plane of the page. The map is scaled to 100 and contoured every 10 relative units starting at 10.0. On the κ = 102° section, the strong peak lies at ω = 90° and ϕ = 45°; there are no other prominent peaks on the 60° or 72° κ sections, which would indicate the presence of sixfold or fivefold elements of symmetry, respectively. (*B*) Height of the ω = 90° and ϕ = 45° peak as function of κ, in steps of 3°. The maximum of each peak is clearly at multiples of 51.4°, indicating the presence of a sevenfold axis of rotational symmetry.

Since the peaks on the κ sections reach maxima at intervals of 51°, and clearly not at intervals of 72° or 60°, the identification of the noncrystallographic sevenfold axis of symmetry, and not a fivefold or sixfold axis, is unambiguous. The orientation of the sevenfold axis of symmetry, as determined from the self-rotation function, is perpendicular to the *b* axis and is ≈45° from the *a* axis. These results agree with the predicted location of the noncrystallographic axis of symmetry based on a streak of intense reflections observed on screened precession photographs of the *h0l* zone.

The data from the self-rotation function (see Fig. 1) do not strictly rule out the combination of a rotation and a translation (i.e., a screw axis) giving rise to the apparent sevenfold axis of rotational symmetry. If there is a screw axis along the noncrystallographic axis of symmetry, then one of the possible conclusions is that the oligomer only displays sevenfold rotational symmetry in a projection along the screw axis. Nevertheless, the combination of the crystallographic and the chemical modification data (see below) provide strong evidence that the αHL oligomer is indeed a heptamer with sevenfold rotational symmetry.

Chemical Modification and Gel-Shift Electrophoresis. The subunit stoichiometry of αHL oligomers formed on rRBCMs has also been reevaluated by an approach based on gel-shift electrophoresis, which has been used previously to count the number of cysteine residues in individual polypeptide chains (38) and to analyze the results of surface labeling experiments on single-cysteine mutants (M. Krishnasastry, B. Walker and H.B., unpublished results). We have adapted the technique to count the number of subunits in the αHL pore by exploiting earlier findings: αHL oligomers are stable during electrophoresis in SDS (39); radiolabeled αHL made by IVTT assembles spontaneously into target membranes (31); and significant shifts in electrophoretic mobility are observed when both monomeric and oligomeric single-cysteine mutants of αHL are modified by the dianionic sulfhydryl reagent IASD (M. Krishnasastry, B. Walker and H.B., unpublished results).

In one variation of our experiment, derivatives of a single-cysteine mutant of αHL, T292C, that had been separately modified with the neutral IAM or with the dianionic IASD were mixed in various ratios (e.g., 12:0, 10:2, 6:6, 2:10, 0:12). The polypeptides in each mixture were then allowed to oligomerize on rRBCMs, and the αHL heteromers thus formed were separated by extended SDS/PAGE. If the oligomeric form contained *n* subunits, we expected to see *n* + 1 bands, representing all possible combinations of the two classes of subunit (namely, α_n, $\alpha_{n-1}\beta_1$, $\alpha_{n-2}\beta_2$ . . . $\alpha_1\beta_{n-1}$, β_n). Electrophoresis revealed eight bands, which suggests that there are seven subunits (Fig. 2*A*). This interpretation presumes that each time a subunit is replaced with an IASD-modified polypeptide, the latter makes an approximately uniform contribution to the shift in electrophoretic mobility, which is independent of the arrangement of the two classes of subunits about the central axis of symmetry.

Chemical modification was also carried out after assembly. WT-αHL and T292C were mixed in different ratios (e.g., 12:0, 10:2, 6:6, 2:10, 0:12), allowed to oligomerize on rRBCMs, and then subjected to extensive modification with IASD. SDS/PAGE and autoradiography revealed eight species, again indicating the existence of a seven-subunit oligomer (Fig. 2*B*).

As well as producing a readily counted ladder of nearly uniformly spaced bands, the present technique for determining subunit stoichiometry has several advantages compared with chemical cross-linking. Notably, it can be applied directly to cell membranes, without the fear that the target

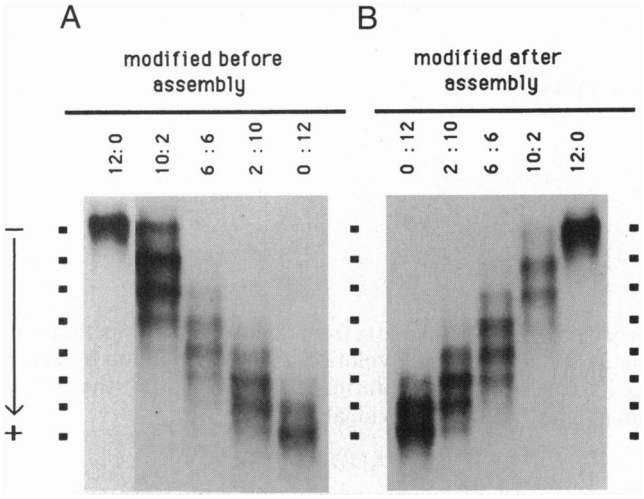

FIG. 2. αHL forms heptamers on rRBCMs as demonstrated by gel-shift electrophoresis. (*A*) Premodified αHL polypeptides form heptamers. ^{35}S-labeled T292C was modified with IAM and IASD in separate reactions. The two modified subunits were mixed in different ratios and allowed to oligomerize on rRBCMs. The oligomers were separated by SDS/PAGE and visualized by autoradiography. Eight species with a molecular mass of ≈200 kDa can be seen, indicating that the αHL oligomers contain seven subunits. The ratio of IAM-modified to IASD-modified polypeptide is indicated above each lane. The first lane is shown at one-third of the exposure time of the remaining four lanes. The relative numbers of counts recovered in this region of the gel were 12:0, 100; 10:2, 45; 6:6, 23; 2:10, 27; 0:12, 22. These values most likely reflect the reduced ability of IASD-derivatized T292C to oligomerize. The latter may also be responsible for the remaining $\alpha_1\beta_6$ in the 0:12 lane, which is presumably enriched for the underivatized T292C that was present at <5% in the reaction mix. (*B*) Unmodified αHL polypeptides form heptamers as detected by chemical modification after assembly. WT-αHL and T292C were mixed in different ratios and allowed to oligomerize on rRBCMs. The membranes were recovered and subjected to extensive modification with IASD. SDS/PAGE and autoradiography revealed eight species, indicating the existence of seven subunits. The ratio of WT-αHL to T292C added to the membranes is indicated above each lane. Under the conditions used for IASD modification, which were designed for selective derivatization of sulfhydryls in the presence of other protein nucleophiles, the derivatization of oligomers formed by T292C alone (lane 0:12) was not quite complete, perhaps due to steric or electrostatic hindrance to attachment of a seventh group.

protein will become cross-linked to other membrane proteins.

For more than 25 years, the αHL pore has been generally regarded as a hexamer. Here, it is demonstrated that the pore is actually a heptamer in both single crystals and on the erythrocyte membrane. Perhaps the previous findings can be explained by preconception together with limitations of the accuracy of the techniques employed. Besides clarifying a fundamental issue concerning the structure of αHL, our approach may have important practical consequences.

This paper is dedicated to the memory of Howard S. Tager. We thank Barbara Walker (Worcester Foundation) for advice and materials and Linda Musil for stimulating discussions. We are very grateful to the laboratories of Keith Moffat and R. Michael Garavito for helpful comments, advice, assistance, and generous use of equipment. This study was supported by grants from the U.S. Public Health Service (National Institutes of Health shared instrumentation Grant RR 06568), the Office of Naval Research (J.E.G. and H.B.), the Department of Energy (H.B.), the American Cancer Society, Massachusetts Division (H.B.), the American Cancer Society, Institutional Grant (J.E.G.), and the Cancer Research Foundation (J.E.G.). This work was partially supported by the Martin D. and Virginia S. Kamen Sustaining Fund for Young Faculty.

1. Ludwig, D. S., Ribi, H. O., Schoolnik, G. K. & Kornberg, R. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8585–8588.
2. Spangler, B. D. & Westbrook, E. M. (1989) *Biochemistry* **28,** 1333–1340.
3. Green, M. J. & Buckley, J. T. (1990) *Biochemistry* **29,** 2177–2180.
4. Wilmsen, H. U., Leonard, K. R., Tichelaar, W., Buckley, J. T. & Pattus, F. (1992) *EMBO J.* **11,** 2457–2463.
5. van der Goot, F. G., Ausio, J., Wong, K. R., Pattus, F. & Buckley, J. T. (1993) *J. Biol. Chem.* **268,** 18272–18279.
6. Unwin, P. N. T. & Ennis, P. D. (1984) *Nature (London)* **307,** 609–613.
7. Musil, L. S. & Goodenough, D. A. (1993) *Cell* **74,** 1065–1077.
8. Bhakdi, S., Füssle, R. & Tranum-Jensen, J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5475–5479.
9. Tobkes, N., Wallace, B. A. & Bayley, H. (1985) *Biochemistry* **24,** 1915–1920.
10. Walker, B., Krishnasastry, M., Zorn, L. & Bayley, H. (1992) *J. Biol. Chem.* **267,** 21782–21786.
11. Walker, B., Krishnasastry, M. & Bayley, H. (1993) *J. Biol. Chem.* **268,** 5285–5292.
12. Walker, B. & Bayley, H. (1994) *Protein Eng.* **7,** 91–97.
13. Walker, B., Kasianowicz, J., Krishnasastry, M. & Bayley, H. (1994) *Protein Eng.* **7,** 655–662.
14. Bhakdi, S. & Tranum-Jensen, J. (1991) *Microbiol. Rev.* **55,** 733–751.
15. Ikigai, H. & Nakae, T. (1985) *Biochem. Biophys. Res. Commun.* **130,** 175–181.
16. Ward, R. J., Palmer, M., Leonard, K. & Bhakdi, S. (1994) *Biochemistry* **33,** 7477–7484.
17. Arbuthnott, J. P., Freer, J. H. & Bernheimer, A. W. (1967) *J. Bacteriol.* **94,** 1170–1177.
18. Olofsson, A., Kavéus, U., Thelestam, M. & Hebert, H. (1988) *J. Ultrastruct. Mol. Struct. Res.* **100,** 194–200.
19. Ward, R. J. & Leonard, K. (1992) *J. Struct. Biol.* **109,** 129–141.
20. Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T. & Wellensiek, H.-J. (1981) *J. Cell Biol.* **91,** 83–94.
21. Olofsson, A., Kavéus, U., Thelestam, M. & Hebert, H. (1992) *J. Struct. Biol.* **108,** 238–244.
22. Hebert, H., Olofsson, A., Thelestam, M. & Skriver, E. (1992) *FEMS Microbiol. Immunol.* **105,** 5–12.
23. Baron, C. & Thompson, T. E. (1975) *Biochim. Biophys. Acta* **382,** 276–285.
24. Garavito, R. M. & Rosenbusch, J. P. (1980) *J. Cell Biol.* **86,** 327–329.
25. Michel, H. & Oesterhelt, D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1283–1285.
26. Brzozowski, A. M. (1993) *Acta Crystallogr. Sect. D* **49,** 352–354.
27. Messerschmidt, A. & Pflugrath, J. W. (1987) *J. Appl. Crystallogr.* **20,** 306–315.
28. Kabsch, W. (1988) *J. Appl. Crystallogr.* **21,** 916–924.
29. Fox, G. C. & Holmes, K. C. (1966) *Acta Crystallogr.* **20,** 886–891.
30. Science and Engineering Research Council (1990) CCP4, Collaborative Computing Project No. 4 (Daresbury Lab., Warrington, U.K.).
31. Walker, B., Krishnasastry, M., Zorn, L., Kasianowicz, J. & Bayley, H. (1992) *J. Biol. Chem.* **267,** 10902–10909.
32. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
33. Hunkapiller, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91,** 227–236.
34. Matthews, B. W. (1968) *J. Mol. Biol.* **33,** 491–497.
35. Michel, H. (1982) *J. Mol. Biol.* **158,** 567–572.
36. Forst, D., Schülein, K., Wacker, T., Diederichs, K., Kreutz, W., Benz, R. & Welte, W. (1993) *J. Mol. Biol.* **229,** 258–262.
37. Rossmann, M. R. & Blow, D. M. (1962) *Acta Crystallogr.* **15,** 24–31.
38. Creighton, T. E. (1980) *Nature (London)* **284,** 487–489.
39. McNiven, A. C., Owen, P. & Arbuthnott, J. P. (1972) *J. Med. Microbiol.* **5,** 113–122.