Crystal Structure and Subunit Dynamics of the Abalone Sperm Lysin Dimer: Egg Envelopes Dissociate Dimers, the Monomer Is the Active Species

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Abstract. Lysin is a 16-kD acrosomal protein used by abalone spermatozoa to create a hole in the egg vitelline envelope (VE) by a nonenzymatic mechanism. The crystal structure of the lysin monomer is known at 1.9 Å resolution. The surface of the molecule reveals two tracks of basic residues running the length of one surface of the molecule and a patch of solvent-exposed hydrophobic residues on the opposite surface. Here we report that lysin dimerizes via interaction of the hydrophobic patches of monomers. Triton X-100 dissociates the dimer. The crystal structure of the dimer is described at 2.75 Å resolution. Fluorescence energy transfer experiments show that the dimer has an approximate \( K_D \) of 1 \( \mu \)M and that monomers exchange rapidly between dimers. Addition of isolated egg VE dissociates dimers, implicating monomers as the active species in the dissolution reaction. This work represents the first step in the elucidation of the mechanism by which lysin enables abalone spermatozoa to create a hole in the egg envelope during fertilization.

During fertilization, spermatozoa must penetrate the extracellular investments surrounding the egg before the plasma membranes of the two gametes can make contact and fuse. To accomplish this, most animal species have evolved sperm with acrosomal vesicles that undergo exocytosis to release lytic proteins used in penetration of the egg coat. Abalone spermatozoa possess an enormous acrosomal vesicle (Lewis et al., 1980). These sperm bind to the elevated egg vitelline envelope (VE) and release concentrated 16-kD lysin from the exocytosing acrosome. Lysin creates a hole in the VE by a nonenzymatic mechanism to allow the sperm cell to pass through the VE and fuse with the egg (Lewis et al., 1982).

Purified lysin exhibits species-selectivity in the dissolution of isolated VE. For example, 12 \( \mu \)g of red abalone (Haliotis rufescens) lysin will dissolve 95% of red abalone egg VE, whereas 12 \( \mu \)g of lysin from the black abalone (H. cracherodii) is ineffective against red abalone VE. Red abalone lysin is also ineffective at dissolving pink abalone (H. corrugata) VE (Vacquier and Lee, 1993). The seven species of Eastern Pacific (California Coast) abalone have overlapping habitats and breeding seasons. The species-selectivity of lysin in dissolving the egg VE may be one mechanism that has evolved to establish prezygotic reproductive isolation among abalone species (Vacquier and Lee, 1993; Lee, 1994; Lee et al., 1995). To cell biology, the species-selective interaction between abalone sperm lysin and the egg VE represents a unique experimental model for studying molecular recognition between two cells.

The deduced amino acid sequences of lysin (mature lysins are 126-138 residues) from 20 species of abalones from various global locales show that the NH\(_2\)-terminal domain of residues 1-12 is always species unique, suggesting that it is the domain of lysin most important in species-selective recognition of the VE (Lee et al., 1995). Monomeric lysin is the first fertilization protein whose crystallographic structure has been solved (Shaw et al., 1993). Resolved at 1.9 Å, several striking features are seen. The monomer is composed of a tight bundle of \( \alpha \)-helices forming a novel fold with no \( \beta \) sheet and no binding pocket or cleft. The species-unique domain of residues 1-12 extends away from the helical bundle. Two parallel tracks of basic residues (1 of 9 and the other 14 residues) run the length of one surface of the protein and the opposite surface possesses a patch of 11 solvent-exposed hydrophobic residues (Shaw et al., 1993). The presence of the basic tracks and the hydrophobic patch suggested the hypothesis that lysin

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may act by competitively disrupting both hydrogen bonds and hydrophobic interactions among VE glycoproteins.

Here we report that at physiological concentrations, lysin exists as a homodimer by interaction of apposing hydrophobic patches of monomers. The crystal structure of the dimer resolved at 2.75 Å is described. Fluorescence energy transfer experiments show that the KD for dimer formation is ~1 μM and that monomers exchange rapidly between dimers. Addition of isolated egg VE results in the dissociation of dimers. The data are consistent with the hypothesis that the monomer is the active species in the dissolution of egg envelopes during fertilization.

Materials and Methods

Isolation of Lysin, Radiiodination and Chemical Cross-linking

Lysin was isolated from spermatozoa of the red abalone, *Haliotis rufescens*, by mincing dissected testes in seawater (all seawater was passed through a 0.45-μm filter), filtering the cell suspension through cheesecloth and then dialyzing the supernatant solution against seawater at 1,000 g for 20 min (4°C). The cells were suspended in 25 vol of seawater containing 0.5% vol/vol Triton X-100 that extracts the acrosomal proteins (30 min). After centrifugation at 30,000 g for 30 min the supernatant was dialyzed against a minimum of 100 vol of 250 mM NaCl/10 mM morpholinooethylsulfonic acid (MES), pH 6.0 (NaCl/MES). After dialysis, the sample was centrifuged as above and the supernatant loaded onto a 60 ml bed (2.5-cm-diam) of CM-cellulose equilibrated in NaCl/MES. The column was washed with at least 10 vol NaCl/MES containing 1% vol/vol Triton X-100/1% vol/vol mercaptoethanol, followed by 10 vol of NaCl/MES containing 3 M urea, and finally a minimum of 20 vol of NaCl/MES alone. The column was eluted so that 5 μl contained 1 μg of lysin that had ~250,000 cpm. To radioiodinate lysin, two ml of a seawater solution of lysin at 1 mg/ml were placed in a round bottom 50-ml plastic tube and 2 ml of carrier-free Na125I (Amersham Corp., Arlington Heights, IL) added. Two iodobeads® (Pierce Chemical Co., Rockford, IL) were added and the tube rocked for 10 min at 23°C. The labeled lysin was dialyzed extensively against seawater/0.04% sodium azide to remove free iodide. The labeled protein retained its ability to dissolve isolated egg VE. The specific activity was diluted so that 5 μl contained 1 μg of lysin that had ~250,000 cpm.

Chemical cross-linking was used to show that lysin is a dimer in solution and that Triton X-100 and other detergents dissociate the dimer. 25 μl portions of seawater were placed in a series of 1.5-ml microfuge tubes. 25 μl of a 1% vol/vol solution of Triton X-100 in seawater was titered down in 10-min exposures per frame. A total of 91 frames were collected in 18 h without appreciable crystal decay. The data were indexed, integrated, merged, and scaled with MAREXS (Kabsch, 1988). For 24,754 observations of 7,682 unique reflections the average I/σ(I) was 44.4 and Rsymm (I) 5.4%. For the shell 2.8-2.6 Å, the average I/σ(I) was 11.3 and Rsymm (I) 15.2%. The data were 78.9% complete to 2.6 Å and 95.6% complete to 2.8 Å resolution.

The structure was solved by molecular replacement using the Xplor suite of programs (Brünger et al., 1989). The 1.9-Å resolution refined structure of monomeric red abalone sperm lysin was used as a search model (Shaw et al., 1993). The rotation function and Patterson correlation refinement gave two clear solutions, corresponding to each monomer in the dimer. The translation functions for each solution gave 14τ (monomer A) and 10τ (monomer B) peaks. As the two translation solutions were of different peak heights, PC refinement was performed using the dimer model with each monomer being refined as a rigid body. The subsequent translation function for the dimer gave a 21σ peak. Analysis of the packing of the molecular replacement solution showed no bad contacts of the monomers and no bad contacts of symmetry-related molecules. The molecular replacement parameters were optimized by rigid body refinement using data between 10.0-6.0 and 8.0-3.0 Å resolution, resulting in an R-factor of 0.425 for all data with I/σ(I)>3.0σp.

The molecular replacement model was refined by positional and simulated annealing using Xplor (Brünger et al., 1989). Positional refinement reduced the R-factor to 25.0% and restrained isotropic individual B-factor refinement further reduced the R-factor to 0.235 for data in the range 8.0-3.0 Å resolution. It was apparent at this stage that the B-factors of monomer B were uniformly higher than for monomer A. To avoid the possibility that close contacts at the dimer interface were preventing correct refinement, residues Arg112, Tyr115, Phe116, Phe118, Asn119, Asn120, Met110, Tyr113, and Tyr117 were truncated to alanine. Simulated annealing refinement was performed at 1,000K using 8.0-2.75 Å resolution data with noncrystallographic symmetry restraints applied to the helices of each monomer. A 2F0-Fc/|Fc| difference Fourier map based on this model, unbiased at the 18 truncated side chains (nine in each monomer), revealed clear density for 16 of the side chains. The truncated side chains were rebuilt using the program suite Xtalview (McRee, 1993). The 2F0-Fc/IFc map was used to adjust the model for other residues in each monomer wherever necessary, as the conformation of some of the loops and surface side chains differed in molecule A versus molecule B, and/or differed with respect to the starting model. The model was then refined by simulated annealing as above, followed by restrained B-factor refinement. A subsequent 2Fo-Fc/IFc map revealed density for the two truncated side chains of residue Lys133, and density was apparent for residues 1-9, 135 and 136 at the NH2 and COOH termini of each monomer. In addition, Met134 is modeled as alanine in each monomer. This map was also used to check and adjust the fit of residues having high B-factors before final simulated annealing and B-factor refinement.

The molecular replacement solution was independently confirmed with a heavy atom derivative. A crystal of dimeric lysin was soaked 18 h in 0.1 mM KAucI4 in a synthetic mother liquor consisting of the reservoir solution used for crystallization. A complete data set to 2.85 Å resolution was collected and processed as for the native data. The average isomorphous difference was 28% on F. The difference Patterson map calculated with data to 4.0 Å resolution contained Harker vectors at u = 0, v = 0, and w = 0.5 (4r, 4r, 6r) consistent with a single site and confirming the space group as P2221. Refinement of the space group using Xcarg (McRee, 1993) at 3.0 Å resolution yielded Rcentric of 0.66 and an average phasing power of 1.63. The refined coordinates of the site (0.0129, 0.3915, 0.8821) are consistent with an isomorphous difference Fourier map calculated using phases from the refined model that contains a 14τ peak at ~0.00, 0.40, 0.88, and no other peaks greater than 3σ in the asymmetric unit. The AucI4 anion binds on the local twofold axis between monomers of the asymmetric unit in a hydrophobic pocket formed by the side chains of residues Phe115, Phe116, Leu120, Met110, Phe117, and Met110 of each monomer. Charge neutralization is provided by the side chain of Arg86 of monomer?

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B that has contact distances of 4.5 and 5.0 Å to the Au site. The only other contacts to the Au site less than 5.0 Å are from Pro12 of each monomer and the carbonyl of Met110 of monomer A. Therefore, the Au site is consistent with the difference Patterson map and a difference Fourier map using model based phases. Furthermore, the site is chemically reasonable with respect to the protein structure.

The final model has a R-factor of 0.233 for all 7328 reflections in resolution range 8.0-2.75 Å with IFI > 0. The model is comprised of residues 10-134 of each monomer (2,096 atoms total). No solvent molecules have been modeled. The rms deviations from ideality of bonds, angles and planes are 0.023 Å, 4.05°, and 1.73°. As noted below, the average B-factor for molecule A is reduced due to crystal packing contacts. Except for three glycines, there are no outliers in the phi plot. Coordinates have been deposited with the Protein Data Bank (Accession Code 1LYN).

**Labeling Lysin with Fluorescent Probes and Fluorescence Energy Transfer Experiments**

TRITC and FITC were from Molecular Probes (Eugene, OR). 1 mg of TRITC was dissolved in 200 μl dimethylsulfoxide (11.25 mM stock). A 950-μl aliquot of lysin in natural seawater (pH 7.8) at 3.86 mg/ml was placed in 1.5 ml microfuge tube and 50 μl of stock TRITC added while vortexing (final TRITC concentration = 0.56 mM). 1 mg FITC was dissolved in 257 μl dimethylsulfoxide (10 mM stock). A 900-μl aliquot of lysin in seawater at 3.86 mg/ml was mixed with 100 μl of FITC stock. Both tubes were incubated 3 h at 23°C and then 50 μl of 2 M glycine added. After 30 min the labeled proteins were separated from the free fluorophores on a 1 × 30-cm column of BioGel-P6 (Bio Rad Laboratories, Richmond, CA) in seawater. The peak void volume fractions were collected and protein determined by the BCA (Pierce) or Lowry assay. The TRITC bound to lysin was quantified using the molar extinction coefficient at 555 nm of 72,000 and for FITC the molar extinction coefficient at 510 nm of 65,000 (Adams et al., 1991). The labeling stoichiometry was 1.1 mol of TRITC and 0.8 mol FITC per mole lysin monomer. The labeled lysins were kept at 4°C in seawater containing 0.04% sodium azide; they retained their ability to dissolve isolated egg VE (Vasquier and Lee, 1993) with the same potency as the unlabeled controls. Lysin is stable indefinitely when stored in seawater/azide at 4°C. The two labeled lysins were combined at least 7 d before experiments, at equal protein concentrations, in various molar ratios of FITC-lysine to TRITC-lysine. For most experiments a molar ratio of 1 FITC-lysin: 4 TRITC-lysin (one part FITC-lysin plus four parts TRITC-lysin, both at 16 μM) was selected to maximize the probability that FITC-lysine monomers would dimerize with TRITC-lysine monomers. Fluorescence measurements were done at 23°C in an ISS K2 spectrofluorometer in the photon counting mode using a 5-mm path cuvette to minimize inner filter effects due to the absorption of light by the probes.

**Results**

**Lysin Is a Dimer with Readily Exchangeable Subunits**

Cross-linking 125I-lysine with DSP was maximal above 4 μM lysin. Data at 2 μM (33 μg/ml) lysin are shown in Fig 1. The proportion of dimer decreased exponentially below 2 μM lysin. Concentrations of Triton X-100 above 0.03% (0.29 mM; the approximate critical micelle concentration of the detergent) dissociated the dimer (Fig. 1). All detergents tested at concentrations above their critical micelle concentration produced the same results. A control of boiling lysin (at 2 μM monomer) in 1% sodium dodecyl sulfate-seawater, before addition of the cross-linker, showed that ~7% of the radioactivity in the dimer band could be due to the random cross-linking of free monomers. Lysin cross-linked with DSP was inactive at dissolving isolated egg VE.

Subunit interactions between lysin monomers were studied using lysin labeled with fluorescent probes. Direct evidence of dimer formation was obtained from resonance energy transfer from FITC-lysine to TRITC-lysine caused by the close proximity of these two probes within dimers compared to free monomers. Resonance energy transfer was monitored by the decrease in the intensity of the fluorescein peak at 514 nm and the relative increase in the intensity of the rhodamine peak at 573 nm upon dimer formation. The dependence of energy transfer on lysin concentration is shown in Fig. 2 with the spectra normalized to the FITC peak at 514 nm. The energy transfer efficiency decreased, as seen by the decrease in relative intensity at 573 nm, when labeled lysin (1F+4R) was diluted serially with seawater from 16 to 0.125 μM, indicating dissociation of the dimers. Below 250 nM the shape of the spectra

![Figure 1. Lysin is a dimer in solution. 125I-Lysin, at 2 μM monomer in seawater, was exposed to the indicated concentrations of Triton X-100 (vol/vol) for at least 30 min and cross-linker added. After 10 min the reaction was stopped with electrophoresis sample buffer. The samples were subjected to SDS-PAGE and autoradiography of dried gels. Triton X-100 dissociates dimers above 0.03%. Similar results were obtained with a variety of detergents. M = monomer; D = dimer. Approximately 0.5 μg lysin protein was loaded per lane (125,000 cpm).](image)

![Figure 2. Effect of lysin concentration on energy transfer between monomers. FITC-lysine and TRITC-lysine (both at 16 μM) were mixed together at a molar ratio of 1 FITC-lysine: 4 TRITC-lysine and diluted with seawater at least 7 d before the experiment. Emission spectra of serial 1:1 dilutions with seawater from 16 to 0.125 μM were recorded (excitation 460 nm). The spectra were normalized to the FITC peak emission. Inner filter effects due to the absorbance of the fluorophores contributed to the increase in the 573 nm peaks at 8 and 16 μM monomer. At or below 4 μM, inner filter effects were negligible. The shape of the spectra below 0.25 μM changed little with concentration, suggesting that nearly complete dissociation had occurred. The spectra from highest to lowest intensity at 573 nm correspond to 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μM lysin monomer.](image)
Information was calculated to be ~1 M. The time course followed a single exponential with a half-time of 7.9 min. Comparison of the emission spectra at equilibrium showed that inclusion of unlabeled lysin decreased the efficiency of energy transfer between FITC-lysin and TRITC-lysin, indicating that unlabeled lysin competes with labeled lysin for dimer formation. The above results demonstrate that lysin dimers dissociate and exchange monomers rapidly.

Crystal Structure of the Dimer

The molecular structure of lysin crystals grown at pH 7.0, refined at 1.9 Å resolution, revealed lysin monomers packed with one molecule per asymmetric unit (Shaw et al., 1993). Association of lysin monomers at neutral pH involves extensive crystal packing contacts between the hydrophobic residues of the extended NH2-terminal domain of one monomer, with a cluster of solvent-exposed hydrophobic residues of an adjacent lysin molecule. However, in the crystals grown at pH 5.5 described herein, the lysin monomers self-associate via the surface cluster of hydrophobic residues to form a lattice with one dimer per asymmetric unit. Previous measurements of dynamic light scattering of solutions of purified lysin at 14 mg/ml (870 µM) at pH 4.5 and 5.5 showed essentially all the lysin to be dimeric with an estimated molecular weight of 38.2 kD (Diller et al., 1994). This result is consistent with the data in Figs. 1 and 2, showing that lysin forms dimers.

The dimer exhibits an extended S shape when viewed along the direction of the local 2-fold axis relating the monomeric helical bundles (Fig 4 a). The overall dimensions of the dimer are ~80 x 40 x 30 Å. There is a cleft ~20 Å wide and 15 Å deep on opposite sides of the twofold axis. When viewed from the side (perpendicular to the local twofold axis) the dimer is essentially flat on one surface, while the opposite surface is convex (Fig. 4 b). When viewed end-on the dimer displays a marked asymmetric clustering of the residues of the hydrophobic patches (Fig. 4 c).

The packing of lysin in the pH 5.5 crystal form is lamellar with dimers forming infinite sheets in the bc plane of the unit cell. The twofold axis relating monomers is approximately normal to this sheet, and the sheets are separated by solvent channels. There are relatively few contacts across the solvent channels. Consequently, the many

Figure 3. Kinetics of lysin subunit dissociation and exchange. (a), Kinetics of lysin dissociation by dilution. 10 µl of 16 µM lysin (1 FITC-lysin + 4 TRITC-lysin; 1F+4R) was added to 490 µl seawater (final concentration 0.32 µM). The time course of the FITC fluorescence change was monitored at 460 nm excitation and 515 nm emission. The solid trace is the experimental data and the dashed line is the fit to a single exponential with rate constant k = 0.53 ± 0.01 min⁻¹ (t₁/₂ = 78 s). (b), Kinetics of lysin subunit exchange. Equal volumes (250 µl) of 8 µM FITC-lysin + unlabeled lysin (1F+4U) and 8 µM TRITC-lysin + unlabeled lysin (4R+1U) were mixed and the time course of the FITC fluorescence change was monitored at 460 nm excitation and 520 nm emission. The solid trace is the experimental data and the dashed line is the fit to a single exponential with rate constant k = 0.088 min⁻¹ (t₁/₂ = 7.9 min).

Figure 4. Three orthogonal views of the crystal structure of dimeric H. rufescens sperm lysin. α-Helices are red and NH2- and COOH-terminal domains are blue. The side chains of 22 Arg and Lys residues of each monomer are shown (C atoms dark blue; N atoms lavender). Arg¹, Lys⁸, and Lys¹⁸ of each monomer are not visible in the crystal structure. The side chains of 11 residues of each monomer that comprise the interacting hydrophobic patches are shown (atoms are colored: C, green; O, red; N, lavender; S, yellow). These 11 residues are Tyr¹, Tyr¹⁸, Leu⁶⁷, Trp⁶⁸, Ile⁶⁹, Ile⁷², Met⁷⁸, Tyr¹⁰⁹, Phe¹¹⁸, Phe¹⁴⁵, and Met¹¹⁰ (Shaw et al., 1993). (a) The view along the local 2-fold axis relating monomers of the dimer. In this view molecule A is to the lower left and molecule B is to the upper right. On molecule A the NH₂ terminus at residue 10 is below the COOH terminus at residue 134 (adjacent to Lys¹³²). (b) The view perpendicular to the local twofold axis, i.e., from the right side of the view in (a), showing that one surface of the dimer is essentially flat, while the opposite surface is convex. (c) End on view of the dimer, i.e., from the bottom of the view in (a), showing that residues of the hydrophobic patches, which participate extensively in the dimer interface, are clustered on one side of the dimer. This feature of the structure is also seen in (b). The figures were made with the program Molscript.

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positively charged residues on the surface of the dimer are shielded from each other in the crystal. There are two packing contacts involving acidic residues. One involves the side chains of Asp$^{128}$ on symmetry-related molecules, and the other involves Glu$^{16}$ and Glu$^{20}$ on symmetry-related molecules. Presumably, these contacts are able to occur at pH 5.5, but not at pH 7.0, explaining why dimers are not observed in crystals grown at pH 7.0.

The individual molecules of the lysin dimer (A and B) have different crystal packing environments. Molecule A has nine contacts (salt bridges or hydrogen bonds) involving 10 residues to symmetry-related A or B molecules via two of the crystallographic twofold axes, whereas molecule B has six such contacts involving five residues and only one crystallographic twofold axis. Consequently, molecule B, having relatively few contacts compared to A, is incorporated into the lattice primarily by virtue of interaction with molecule A. Consistent with the crystal packing environment, the average temperature factor for molecule B is higher than for molecule A, 20.3 Å$^2$ versus 13.2 Å$^2$. By comparison, the average temperature factor for monomeric lysin (M) in crystals grown at pH 7.0 is 20.9 Å$^2$ (Shaw et al., 1993), suggesting that the average temperature factor of molecule A is reduced due to constraints of the crystal lattice.

Comparison of the structures of A, B, and M molecules shows that lysin has a relatively rigid helical bundle structure with flexible loops and connecting chain segments, and highly flexible NH$_2$ and COOH termini.

Least square fit of all Ca atoms results in the following rms differences: A vs. M, 0.69 Å; A vs. B, 0.61 Å; B vs. M, 0.57 Å. However, least squares fit of 88 Ca atoms in the five α-helices yields rms deviations in the range of 0.39–0.49 Å for these three comparisons, showing that the helical bundle is very similar in all three molecules. Residues that differ at Ca by more than two-times the rms difference predominantly reside in the interhelical segments and loops, including residues 10, 11, 38–40, 74, 75, 78, 79, 96–100, 107–110, and 132–134. The side chain conformations of some of these residues also differ significantly, in particular for Phe$^{10}$, His$^{38}$, Arg$^{40}$, Ile$^{68}$, Asp$^{97}$, Tyr$^{100}$, Phe$^{104}$, Lys$^{106}$, Met$^{110}$, and Tyr$^{130}$. In addition, the NH$_2$-terminal residues 1–9 are completely disordered in the lysin dimer, while residues 4–9 remain ordered in the monomer. The COOH-terminal residues Gly$^{135}$ and Lys$^{138}$ are disordered in the structures of A, B, and M molecules. Temperature factors are also consistently higher for the interhelical and terminal regions compared to helical segments.

Lysin dimerizes via its exposed hydrophobic patch. Altogether, 12 residues of each monomer are involved in contacts of less than 3.5 Å at the dimer interface (Fig. 5 a). The interactions primarily involve hydrophobic and aromatic side chains. The central feature of the interface is a stack of aromatic residues in which Phe$^{101}$ and Phe$^{104}$ of molecule A and B intercalate with each other. Listing them in order, the stack is comprised of His$^{61}$, Tyr$^{57}$, Phe$^{101}$, Phe$^{104}$, Phe$^{104}$, Phe$^{101}$, Tyr$^{57}$, and His$^{61}$ (prime indicates residues on molecule B; see Fig. 5 b). The intercalation of Phe$^{104}$ between Phe$^{101}$ and Phe$^{104}$ resembles the stacking of Phe$^{10}$ on Phe$^{101}$ between monomers (Shaw et al., 1993). Flanking this stack are Met$^{110}$, Met$^{110}$, Tyr$^{100}$, Tyr$^{100}$, Lys$^{106}$, and Lys$^{106}$. The Tyr and Lys side chains shield the central four Phe residues of the stack from solvent. Additional hydrophobic contacts involve the side chains of Lys$^{113}$, Tyr$^{117}$, Lys$^{113}$ and Tyr$^{117}$. There are only two hydrogen bonds in the dimer interface, one between the side chain of Arg$^{80}$ and the carbonyl of Ile$^{111}$ (2.85 Å) and the other between the side chain of Lys$^{108}$ and the carbonyl of Asp$^{97}$ (2.68 Å). The reciprocal interactions on the opposite side of the dimer 2-fold axis cannot occur because the contact distances are greater than 5 Å. Polar contacts are less than 3.5 Å involving His$^{61}$, Lys$^{108}$, and Asn$^{109}$ occur, but do not have the proper geometry for hydrogen bonding. Altogether, of 20 contacts at the dimer interface, 14 are hydrophobic or stacking interactions, two are specific hydrogen bonds and four are unspecified pola interactions. These contacts are not distributed symmetrically: there are 15 for molecule A to molecule B, but only five for molecule B to molecule A on the opposite side of the dimer twofold axis. The asymmetry is accommodated by alternate conformations of the side chains, and by the
fact that most of the residues involved reside on, or are adjacent to, the flexible interhelical segments of residues 95-99 and 107-116. The asymmetry may result from the different crystal packing environments of A and B molecules.

A central feature of the dimer interface is the association of five residues, Tyr57, Tyr100, Phe104, Phe106, and Met110, within the hydrophobic patch of each monomer, which shields their side chains from solvent (Fig. 5 b). The total surface area buried per monomer due to dimerization is 669 Å² as computed with a 1.4-Å probe sphere (Connolly, 1983). Of this total area, 276 Å², or 41% per monomer, arises from these five residues alone. The remaining residues of the hydrophobic patch contribute only 5 Å² per monomer to the total buried surface area. Consequently, in the lysin dimer a significant portion of the hydrophobic patch remains exposed (~310 Å² per monomer). Thus only about half of the hydrophobic patch is involved in forming the dimer. Other residues involved in the interface, in addition to the above mentioned five, contribute another 191 Å², or 29%, of the total buried surface area. The remaining 30% (202 Å²) represents cavities within the dimer interface that are inaccessible to the 1.4-Å probe sphere.

The lysin dimer interface is fairly typical of other protein-protein interactions both in terms of total surface area and the proportion of polar and nonpolar residues involved, although there are only two hydrogen bonds in the lysin dimer (Janin and Chothia, 1990; Chacko et al., 1995). An unusual feature of the lysin dimer interface is the intercalation of Phe104, Phe106, Phe109, and Phe111, which provides specificity to the interaction of monomers due to shape complementarity. A dimer interface with this characteristic, i.e., complementary surfaces of hydrophobic residues, is observed in the dissociable dimer of Chromatium vinosum cytochrome c (Ren et al., 1993). The specific interaction of the Phe residues in the lysin dimer also resembles the interaction of human growth hormone with its receptor. In this case two Trp residues of the receptor and to a lesser extent three Ile and a Pro at the center of the interface, are involved in contacts that provide the majority of the binding energy stabilizing the complex (Clackson and Wells, 1995). By analogy, one could expect that the majority of binding energy in the lysin dimer arises from intercalation of the Phe103 and Phe105 residues, in combination with the association of Tyr57, Tyr100, and Met110 residues of each monomer.

Red abalone sperm lysin is a remarkably basic protein with 12 Arg and 13 Lys residues out of 136 amino acids (Vacquier et al., 1990). In the structure of the monomeric protein, 23 of these residues are arranged in two separate, approximately parallel, tracks running the length of the molecule (Shaw et al., 1993). In the structure of the dimer, 22 of these basic residues are ordered in each monomer, resulting in an array of 44 Arg and Lys side chains on the surface of the dimer (Fig. 6). The basic tracks lie on opposite sides of the dimer. Furthermore, they are approximately equidistant from the dimer interface. The arrangement of the basic tracks in the dimer is apparent in Fig. 4 c. As viewed in Fig. 6, the tracks comprised of residues 29, 33, 36, 71, 72, 78, 87, 94, and 95 lie toward the front of each monomer, and those comprised of residues 13, 20, 40, 47, 48, 55, 106, 123, and 125 lie toward the back. Four residues associated with this latter group, Arg56, Lys108, Lys113, and Lys136 are offset from the essentially linear array of the rest of the side chains in this track; they lie closer to the dimer interface. In fact, the first three of these residues participate in dimer interface contacts. Arg1, and Lys136 (the two termini of each monomer) and Lys9 are not visible in the crystal structure of the dimer. These residues could be expected to be juxtaposed to the basic tracks at the back of the dimer as viewed in Fig. 6. Including these residues, the lysin dimer has a net charge at pH 7 of +26.

### Vitelline Envelopes Dissociate Lysin Dimers

The addition of VE to lysin dimers labeled with both probes (1F+4R) resulted in a concentration dependent loss of energy transfer as seen 10 min after VE addition by the decrease in the relative intensity of the TRITC peak at 573 nm (Fig. 7). The time course of the VE-induced loss of energy transfer was monitored as the increase in FITC emission at 520 nm; it followed a single exponential with a rate constant of 1.4 min⁻¹ (Fig. 8). These data show that the VEs dissociate lysin dimers and suggest that the monomer is the active species in the dissolution reaction. Furthermore, fluorescence polarization measurements indicated that the rotational motion of monomers labeled with either fluorophore decreased upon addition of VEs, suggesting that the monomer was bound tightly to VE protein (data not shown). These data are in accord with the original observation that lysin binding to the VE molecules is an irreversible process (Lewis et al., 1982).

### Discussion

When the structure of monomeric red abalone sperm lysin was resolved it revealed three striking features associated with its surface: two tracks of basic residues, a large, exposed hydrophobic patch, and the clustering of residues that are hypervariable among species (Shaw et al., 1993, 1994). This paper presents the crystal structure of the ho-
The presence of the hydrophobic patch on the opposite surface of the monomer from the basic tracks imparts to lysin a net hydrophobic dipole, or amphipathic character. The amphipathic character of lysin imparts on the protein the ability to be a potent fusagen of artificial lipid vesicles (Hong and Vacquier, 1986). While this property may be utilized lysin from the red abalone (H. ruforescens), lysin from two other California species (H. cracheroidii and H. corrugata) also exist in solution as dimers and yield the same cross-linking pattern as that shown in Fig. 1.

This paper provides new information about the function of lysin by showing that it is a dimer in solution that monomerizes during, or immediately after, its contact with the VE. Lysin is a major protein of abalone sperm cells that is released in a highly concentrated form onto the VE (Lewis et al., 1982). The exact concentration of lysin on the VE at the point of sperm contact is unknown, but certainly it is greater than 62 μM (1 mg/ml). Measurements of dynamic light scattering of lysin at 14 mg/ml (870 μM) showed lysin to be a dimer (Diller et al., 1994). At much lower lysin concentrations, the cross-linking and fluorescence energy transfer data also show that lysin is a dimer. The data indicate that monomers exchange rapidly between dimers (Fig. 3), and at concentrations below ~0.25 μM, dimer dissociation is nearly complete (Fig. 2). Most importantly, the fluorescence energy transfer data show that dimers dissociate in the presence of the VE (Figs. 7 and 8).

Because lysin binds the VE as a monomer, or monomerizes after binding, the previous analysis of the surface features of the protein must be considered. The function of the basic tracks may be to cleave hydrogen bonds between glycoprotein molecules (Lewis et al., 1982) comprising the VE, whereas the function of the hydrophobic patch may be to disrupt hydrophobic interactions (Shaw et al., 1993, 1994). Until more is known about the interaction of the VE and lysin, each hypothesis, or their combination, should be considered as plausible. The available data strongly implicate the NH2-terminal domain of residues 1–12 as being responsible for species-selective recognition (Shaw et al., 1993, 1994; Lee et al., 1995). At this time, the remarkable sequence conservation among 20 species of the residues comprising both the basic tracks and the hydrophobic patch (Lee et al., 1995) supports the idea that both these surface features are involved in the dissolution mechanism. Thus, we envision a minimum of three separate interactions between lysin and the VE. The first is mediated by the two NH2 termini of the dimer and are species selective. This species recognition allows for the “proper fit” of the dimer with certain VE molecules; this results in the second step that is the dissociation of the dimer. The third step is mediated by either or both the hydrophobic patch and the basic tracks, the result being that the 13-nmdiam fibers comprising the VE lose cohesion and unravel (Lewis et al., 1982; Mozingo et al., 1995). While the first step shows species-selectivity, steps two and three are general to all abalone sperm lysins. DSP cross-linked lysin is inactive at dissolving egg VE. Although consistent with the idea that the monomer is the active species, DSP would also react with the amino groups of the 13 Lys residues of each monomer and may thus destroy the function of the basic tracks. Although the work in this paper has utilized lysin from the red abalone (H. ruforescens), lysin from two other California species (H. cracheroidii and H. corrugata) also exist in solution as dimers and yield the same cross-linking pattern as that shown in Fig. 1.

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self association of monomers. The structural data argue that this is a stereo specific, rather than random, interaction. The hydrophobic surfaces of the dimer interface have complementary shapes and in particular there is a striking intercalation involving four Phe residues. Thus, an important function for the hydrophobic patch, not apparent from the structure of the monomer alone, is to mediate stereo-specific interaction of monomers.

If the monomer is the active form of lysin, what could be the importance of the existence of the dimeric lysin before its interaction with the VE? One possibility is that in the dimer form, the amphipathic character of lysin is neutralized, providing a water soluble protein that can be delivered in high concentration in a high ionic strength aqueous medium (seawater). An alternative possibility is that dimerization is important in the packaging of lysin in the acrosomal granule (Lewis et al., 1980; Haino-Fukushima and Usui, 1986). The crystal packing structure of the lysin dimer shows that the basic tracks on adjacent dimers are shielded from each other by extensive solvent channels. A similar arrangement in the acrosome would optimize the packing of the very basic molecules at physiological ionic strength.

A third potential reason for the existence of the dimer is implied by its dissociation in the presence of the VE. The dimer could shield the hydrophobic patch from nonspecific interaction with other hydrophobic domains of cellular components. Upon exocytosis of the lysin directly onto the VE (Lewis et al., 1982), the low affinity association of monomers, and molecular recognition of lysin with the VE, results in dimer dissociation and exposure of the hydrophobic patch inside the fibrous matrix of the VE (Mozingo et al., 1995). Dimerization thus may protect the hydrophobic patch until contact between the dimer and the VE is made. Support for this idea comes from the fact that $^{125}$I-lysine binds tenaciously to paraffin coated microfuge tubes. Unlabeled lysin competes with labeled protein in binding to paraffin. Once bound to paraffin, 5% vol/vol Triton X-100 will not displace lysin. However, the presence of low concentrations of Triton X-100 (0.02%) will prevent lysin from binding to paraffin. Once bound to paraffin, 5% SDS will displace the protein from the paraffinized tube (Vacquier, V. D., unpublished data). The hydrophobic patch is the only domain of the lysin molecule that could bind to paraffin since the remainder of the surface of lysin is highly polar. These data indicate that the hydrophobic patch can bind tightly, and nonspecifically, to other hydrophobic molecules. This property may explain why lysin binds irreversibly to VE molecules.

Because Triton X-100 will dissociate dimers we determined the effect of this detergent on the ability of lysin to dissolve VE; concentrations above 0.03% were partially inhibitory. For example, at detergent concentrations between 0-0.02%, 63 µg of lysin were required to dissolve 50% of the isolated VEs, whereas at 0.04%, 105 µg lysin was required. Although showing that the monomer is active in the presence of Triton X-100, such experiments do not distinguish if the inhibitory effect of the detergent involves its binding to the VE, to lysin, or to both. Future experiments will be directed toward identifying the components of the VE to which lysin binds and at understanding the chemical nature of their interaction.

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