Acta Cryst. (1994). D**50**, 620–626

Acid pH Crystallization of the Basic Protein Lysin from the Spermatozoa of Red Abalone (*Haliotis rufescens*)

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(*Received* 29 *October* 1993; *accepted* 26 *November* 1993)

Abstract

A new crystal form of dimeric red lysin, a distinctly basic protein ($M_r = 16070$) from the red abalone (*Haliotis rufescens*), has been obtained using ammonium sulfate as precipitant with a sodium citrate–boric acid–citric acid buffer at pH 4.5. The acid pH crystal form resulted from a study aimed at developing conditions favorable to the sitting-drop vapor-diffusion crystallization of other abalone lysins which do not crystallize at neutral or basic pH conditions. The space group is $P222_1$ with cell dimensions $a = 51.2$, $b = 47.0$, $c = 123.8$ Å and two molecules per asymmetric unit.

Introduction

Lysin is an amphipathic protein released from the acrosomal granule of abalone spermatozoa upon adhesion of the sperm to the glycoproteinaceous vitelline envelope surrounding the egg (Hong & Vacquier, 1986; Vacquier, Carner & Stout, 1990; Shaw, McRee, Vacquier & Stout, 1993). The sperm penetrates the vitelline envelope by a non-enzymatic mechanism preceding sperm–egg membrane fusion in sea water (Hong & Vacquier, 1986; Lewis, Talbot & Vacquier, 1982).

Orthorhombic crystals of monomeric lysin from the red abalone, grown at pH 7.0 with either ammonium sulfate or polyethylene glycol 4000, contain one molecule per asymmetric unit, belong to the space group $P2_12_12_1$, and have unit-cell dimensions $a = 52.3$, $b = 46.0$, $c = 81.5$ Å (Baginsky, Stout & Vacquier, 1990). Examination of the neutral pH red lysin crystal structure (Shaw, McRee, Vacquier & Stout, 1993) indicated that not all amino-acid residues involved in intermolecular packing are conserved in the homologous sequences of the seven California abalone lysins (Lee & Vacquier, 1992) and attempts to crystallize lysins from pink and black (*Haliotis corrugata* and *Haliotis cracherodii*, respectively) abalone have not yielded crystals under similar conditions. However, it is of interest to crystallize lysins from other species because these proteins display hypervariable N-terminal domains which are believed to mediate species-specific molecular recognition by the spermatozoa (Shaw, McRee, Vacquier & Stout, 1993).

Lysins are very basic proteins. The experimentally determined isoelectric point of red abalone lysin is about 9.0 (Vacquier, Carner & Stout, 1990). Amino-acid sequences (Lee & Vacquier, 1992) indicate that lysin from red abalone has an expected net charge at neutral pH of +13 (Shaw, McRee, Vacquier & Stout, 1993); lysins from pink and black abalone have expected net charges of +16 and +20, respectively. Additionally, lysins from the other California abalone species exhibit some cross-species activity and possess >60% overall identity of amino-acid residues in pairwise comparisons with red abalone lysin (Lee & Vacquier, 1992). Therefore, a systematic approach was taken to discover the crystallization conditions which would yield new crystal forms of red abalone lysin. The information obtained from this study of red abalone lysin crystallization parameters will be used to obtain crystals of other lysins which conserve those amino-acid residues involved in crystal-packing interactions.

Strategies have been developed to systematically identify, optimize and characterize the parameters which reproducibly result in the nucleation and

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ISSN 0907-4449

growth of diffraction-quality protein crystals: acid pH crystallization of basic proteins by ion pairing (Riès-Kautt & Ducruix, 1991), screening techniques for determining protein solubility characteristics (Stura & Wilson, 1990, 1992; Stura, Nemerow & Wilson, 1992), and factorial experimental designs (Carter, 1992). Techniques for crystallizing basic proteins by ion pairing are based on a study (Riès-Kautt & Ducruix, 1991) which illustrates the effects different precipitants have on the solubility of the basic protein lysozyme and shows that the main effects at pH 4.5 and 291 K are due to anions in a reverse order of the Hofmeister (1888) lyotropic series: $SCN^- > Cl^- > HCO_3^- >$ citrate^{2-} > $CH_3COO^- \simeq$ phosphate > SO_4^{2-} (Riès-Kautt & Ducruix, 1989, 1992). Ion pairing with thiocyanate ions (Riès-Kautt & Ducruix, 1991) has been successfully applied to the crystallization of the basic proteins lysozyme (Riès-Kautt & Ducruix, 1989), erabutoxin b (Saludjian *et al.*, 1992) and, at low salt concentrations, fasciculine 1 (Ménez & Ducruix, 1990). Thiocyanate ions have been hypothesized to preferentially bind arginine side chains (Pande & McMenamy, 1970) and have been shown to bind human serum albumin 25 times more tightly than chloride ions (Scatchard, Scheinberg & Armstrong 1950*a*,*b*).

Experimental

Lysin preparation

Native lysin from red abalone spermatozoa was isolated and purified as described by Lewis, Talbot & Vacquier (1982). Denatured lysin was first renatured by heating for 30 min at 353 K in 5 *M* guanidine hydrochloride and then dialyzing at 277 K for a total of 76 h, with one change of solution after 8 h, against 40 volumes of 0.2 μm vacuum-filtered sea water consisting of 450 m*M* NaCl, 9.7 m*M* KCl, 9.6 m*M* $CaCl_2$, 26.7 m*M* $MgCl_2$, 28.9 m*M* $MgSO_4$, 0.02%(*w*/*v*) NaN_3 and 2.5 m*M* $NaHCO_3$ with a measured pH of 8.0. Renatured and native lysins behaved identically when subjected to the vitelline envelope dissolution assays and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (described by Lewis, Talbot & Vacquier, 1982). The concentration of each separate lysin preparation, initially stored at 253 K, was determined (Lewis, Talbot & Vacquier, 1982) to be 0.555 mg lysin ml^{-1}. Both the native and renatured preparations were subsequently thawed to 277 K and independently concentrated to 5 mg ml^{-1} by pressure filtration with a 3 kDa exclusion membrane (Amicon). Concentrated protein preparations were then diluted 150-fold with glass-distilled water containing 0.1%(*w*/*v*) NaN_3 and 50 m*M* acetic acid–sodium acetate buffered at pH 5.5 (based on the method of Riès-Kautt & Ducruix, 1991) and independently concentrated at 277 K to produce 11–14 mg protein ml^{-1}, low ionic strength solutions for crystallization experiments. Final protein concentrations were spectrophotometrically determined at 280 nm (Hewlett-Packard 8452A Diode Array extinction coefficient = 2.04 absorbance units ml mg^{-1} cm^{-1}).

Light scattering

Dynamic light-scattering data were collected every 30 s for 6 min at 300.5 K and processed with a molecular size detector (Protein Solutions DynaPro-801) to determine the aggregation state of lysin. Count rates varied between 132 and 148, baselines were between 1.000 and 1.004, and all sums of squares after bimodal curve fitting were < 1.210.

Preparation of solutions

All solutions were prepared using glass-distilled water containing 0.1%(*w*/*v*) NaN_3. Solubility screening stock solutions were prepared as described by Stura & Wilson (1992). The sodium citrate–boric acid–citric acid stock buffer was prepared by mixing 1.5 *M* sodium citrate–10 m*M* boric acid (pH ~8.0) with 1.5 *M* citric acid–10 m*M* boric acid (pH ~1.4) to the desired pH. The 3.0 *M* stock solution of $(NH_4)_2SO_4$ was adjusted using 3.0 *M* H_2SO_4. The phosphate stock solution was mixed to the desired pH using 4.0 *M* K_2HPO_4 and 4.0 *M* NaH_2PO_4 solutions. The ethylenediaminetetraacetic acid (EDTA) stock solution was prepared by mixing 10 m*M* EDTA (free acid) with 10 m*M* EDTA (disodium salt, dihydrate) to a pH of 4.5.

Crystallization procedures

Subsequent to setting up vapor-diffusion experiments at room temperature as outlined by Stura & Wilson (1990, 1992), the 24-well plates (Costar) were stored in a constant temperature incubator (Precision Scientific) at 295.3 ± 0.5 K. One plate was equilibrated at 291 K to determine temperature effects. Each vapor-diffusion chamber contained a 1 ml reservoir and sitting drop. Each sitting drop was formed by adding 2.5 μl of reservoir solution to 2.5 μl of protein solution without mixing (Stura, Nemerow & Wilson, 1992).

Screening

The factorial approach developed by Carter (1992) was modified in setting up the 370 sitting drops (≃11 mg of protein) which cumulatively screened

Table 1. *Qualititative results of screened effectors*

Effector*	Concentrations tested†	Results
Buffer system‡		
Acetate, pH = 4.5	10–40 m*M*	Greater acetate concentrations were required to produce the same effects as citrate
with EDTA, pH = 4.5	10–40 m*M* acetate, 50 and 100 μ*M* EDTA	
Citrate, pH = 4.0–7.0	15–38 m*M*	50 μ*M* EDTA had no effect, 100 μ*M* EDTA promoted growth along *c* dimension and 200 μ*M* EDTA promoted nucleation (acted as co-precipitant)
with EDTA, pH = 4.5	15–38 m*M* citrate; 50, 100 and 200 μ*M* EDTA	
with ammonium acetate, pH = 4.3	22.5 m*M* citrate; 50 and 100 m*M* acetate	No differences noted under tested conditions
Phosphate (NaH_2 and K_2H), pH = 6.0–7.0	0.25, 0.4, 0.55 and 0.7 *M*	See phosphate entry under salts
Additives and precipitants		
Salts		
$(NH_4)_2SO_4$	0.35–0.75 *M*	The use of lithium or sodium sulfate instead of ammonium sulfate inhibited the slow conversion of prismatic crystals to needles
Na_2SO_4	0.6 *M*	
Li_2SO_4¶	0.05, 0.1 and 0.48 *M*	
NaH_2PO_4 and K_2HPO_4	0.25, 0.4, 0.55 and 0.7 *M*	Sitting drops remained clear at 0.25 *M*, contained small crystals at 0.4 *M*, and produced aggregated precipitates ≥0.55 *M*
Acetate, ammonium§	0.48 *M*	Slightly more numerous smaller crystals
Citrate, sodium	0.015–0.70 *M*	Sitting drops remained clear <0.25 *M* and produced precipitation ≥0.45 *M*
Na_2CO_3§	0.05 and 0.1 *M*	Particulate precipitation occurred
$(NH_4)_2CO_3$§	0.05 and 0.1 *M*	Sitting drops remained clear
$(NH_4)SCN$§	0.01–0.048 *M*	Large crystals ≤25 m*M*; granular precipitation at higher concentrations
Benzoate, sodium§	0.001–0.075 *M*	Sitting drops remained clear ≤10 m*M*; ≥15 m*M* produced particulate precipitation
Detergents§		
Triton X-100	0.005 and 0.025%(*v*/*v*)	Did not affect crystal growth or nucleation
LDAO	0.005 and 0.025%(*w*/*v*)	Did not affect crystal growth or nucleation
OTGP	0.005–0.05%(*w*/*v*)	Promoted crystal growth along *c* dimension
Organics§¶		
Glycerol	1%(*v*/*v*)	Solubilized lysin
1-Propanol	1%(*v*/*v*)	Solubilized lysin
MPD	1 and 40%(*v*/*v*)	Solubilized lysin
Dioxane	1 and 2%(*v*/*v*)	Solubilized lysin
Carbohydrates§		
Fucose	0.75 and 2.5 m*M*	Promoted growth at 0.75 m*M* and nucleation at 2.5 m*M*
Fucosylamine	0.25–1.0 m*M*	Inhibited nucleation and reduced growth rate
Dyes§		
Alcian blue, 8GX	0.005–0.05%(*w*/*v*)	Blue, aggregated precipitation occurred at 0.05%; produced disordered, blue-dyed crystals at 0.025%; and resulted in well ordered blue-striped crystals ≤0.01%
Alcian blue, pyridine derivative	0.005–0.05%(*w*/*v*)	Produced more disordered, blue-dyed crystal at 0.05%

* All sitting drops contained 25 m*M* sodium acetate–acetic acid (pH 4.5, 5.3 or 5.5) added from the protein solution.

† Reservoir concentrations were 2× the listed sitting-drop concentrations.

‡ Buffer systems used mixtures of free acids and sodium salts unless noted otherwise, all buffers acted as co-precipitants, and results were compared with those obtained using an equimolar amount of citrate buffer.

§ Precipitants were tested in sitting drops containing 0.5 *M* $(NH_4)_2SO_4$, and results were compared with those obtained using 0.6 *M* $(NH_4)_2SO_4$. Additives were tested in sitting drops containing 0.5 or 0.6 *M* $(NH_4)_2SO_4$ and results were compared with those obtained using 0.6 *M* $(NH_4)_2SO_4$ in the absence of the tested additive.

¶ Each organic was also screened for two-factor interactions with ammonium acetate, ammonium carbonate, sodium carbonate and lithium sulfate in sitting drops containing 0.45 *M* $(NH_4)_2SO_4$.

two temperatures, 15 pH values, six buffer systems, 11 precipitants and 15 additives for their effects on the crystal nucleation and growth of lysin from the red abalone (Table 1). Owing to the successful crystallization of lysin using ammonium sulfate (Baginsky, Stout & Vacquier, 1990), the first 24-well plate consisted of screening four ammonium sulfate concentrations at six pH values (Table 2). Qualitative, not quantitative, scoring was subsequently used to interpret the experimental results. The refined pH and ammonium sulfate concentrations were tested on both native and renatured lysin preparations. Additional screening experiments were designed to identify other nucleation and growth factors important for obtaining lysin crystals suitable for X-ray diffraction analyses.

Table 2. *Scores from the first experiment used to locate optimum conditions supporting the nucleation and growth of a new crystal form of red abalone lysin*

Scores were assigned according to a scale of crystal quality as follows: cloudy precipitate, 1.0; particulate precipitate, 2.0; spherulites, 3.0; needles, 4.0; plates, 5.0; prisms, 6.0 (Carter, 1992). Standard deviations of averaged scores appear in parentheses. Vapor-diffusion sitting drops at tabulated pH levels contained 0.50–0.75 *M* $(NH_4)_2SO_4$. Each 5 μl sitting drop contained equal volumes of reservoir and protein solutions. Crystallization conditions located using this first experiment and techniques described by Stura & Wilson (1992) were refined to optimize conditions for producing diffraction-quality crystals.

	pH						
	4.5	5.0	5.5	6.0	6.5	7.0	
	Average scores for pH levels						Overall pH scores
	6.0 (0)	6.0 (0)	1.62 (0.23)	1.5 (0)	2.0 (2.3)	2.0 (2.3)	3.19 (2.19)
$[(NH_4)_2SO_4]$	Individual scores						Average scores for $[(NH_4)_2SO_4]$
0.75	6.0	6.0	1.5	1.5	2.0	2.0	3.00 (2.12)
0.70	6.0	6.0	1.5	1.5	2.0	2.0	3.00 (2.12)
0.60	6.0	6.0	1.5	1.5	4.0	4.0	3.83 (1.58)
0.50	6.0	6.0	2.0	1.5	0	0	2.58 (2.52)
							Overall $[(NH_4)_2SO_4]$ score
							3.10 (0.52)

X-ray diffraction

The space group was determined from precession photography. Intesity data were collected using Cu $K\alpha$ radiation from an Elliot GX-21 X-ray generator equipped with Franks focusing mirrors (Harrison, 1968). The data were recorded with an MAR image-plate area detector and processed using *XDS* (Kabsch, 1988). Molecular-replacement calculations were performed using *MERLOT* (Fitzgerald, 1988) and the *X-PLOR* suite of programs (Brünger, Karplus & Petsko, 1989).

Results and discussion

Crystals of lysin were reproducibly grown in vapor-diffusion sitting drops at acid pH using several different additives and precipitants. Qualitative results are summarized in Table 1. The conditions of the initial screen produced needles near neutral pH and prismatic crystals at more acidic pH (Table 2). Prismatic crystals which grew at the bottom of sitting drops at acid pH using < 1.2 *M* $(NH_4)_2SO_4$ and no other precipitants in the reservoir dissolved after 4 months and re-formed as needles on sitting-drop surfaces (Fig. 1*a*).

The first crystal of native red lysin suitable for precession camera photographs grew at 295.3 K after 30 d to a size of 0.2 × 0.6 × 0.9 mm in a 5 μl sitting drop. The protein solution consisted of 11 mg lysin ml^{-1} (0.68 m*M*), 50 m*M* sodium acetate–acetic acid buffer (pH 5.5) and 0.1%(*w*/*v*) NaN_3; the reservoir solution contained 1.0 *M* $(NH_4)_2SO_4$, 45 m*M* sodium citrate–citric acid–0.3 m*M* boric acid buffer (pH 4.5), 100 μ*M* EDTA buffer (pH 4.5) and 0.1%(*w*/*v*) NaN_3. The same conditions at 291 K instead of 295.3 K yielded more numerous smaller crystals. This was probably a result of the decrease in solubility of proteins at lower temperature.

Crystals of renatured red lysin also grew in 5 μl sitting drops comprised of equal volumes of protein and reservoir solutions. In this case, the protein solution consisted of 14 mg lysin ml^{-1} (0.87 m*M*), 50 m*M* sodium acetate–acetic acid buffer (pH 5.5), and 0.1%(*w*/*v*) NaN_3; the reservoir solution consisted of 52.5 m*M* sodium citrate–citric acid–0.35 m*M* boric acid buffer (pH 4.5), 100 μ*M* EDTA buffer (pH 4.5), 0.1%(*w*/*v*) NaN_3 and either 1.23 *M* $(NH_4)_2SO_4$ (Fig. 1*b*) or 0.85 *M* $(NH_4)_2SO_4$ with 20 m*M* NH_4SCN (Fig. 1*c*). A 0.1 × 0.4 × 0.5 mm single crystal of renatured lysin (Fig. 1*d*) grew under the same conditions producing the multi-crystalline form depicted in Fig. 1(*c*) except that the reservoir solution was prepared with 0.80 *M* $(NH_4)_2SO_4$ and 20 m*M* NH_4SCN as co-precipitants instead of 0.85 *M* $(NH_4)_2SO_4$ and 20 m*M* NH_4SCN. When a reservoir solution contained 60 m*M* NH_4SCN and no ammonium sulfate, crystals with a distinctly different growth pattern resulted (Fig. 1*e*).

Growth along the shortest crystal dimension was stimulated when either 5 m*M* fucose or 0.01%(*w*/*v*) 1-*S*-octyl-β-D-thioglucopyranoside (OTGP) was used with 1.2 *M* $(NH_4)SO_4$ (no ammonium thiocyanate) in the reservoir. Neither 0.05%(*w*/*v*) Triton X-100 nor lauryldimethylamine oxide (LDAO) in the reservoir appeared to affect the growth of lysin crystals. Alcian blue 8GX (a copper-containing dye) stained lysin crystals (Fig. 1*f*), but the pyridine derivative of alcian blue at an equivalent concentration did not

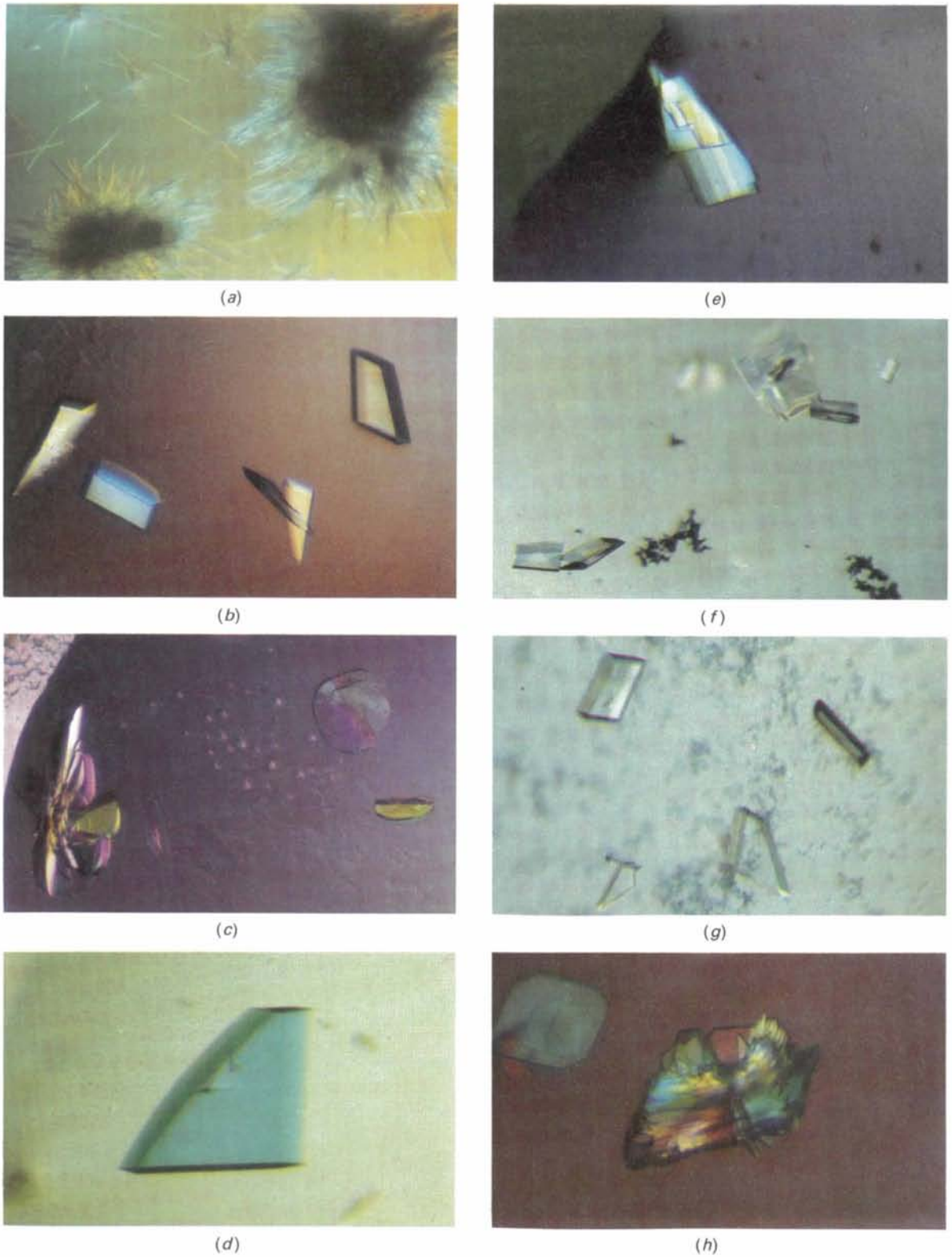

Fig. 1. Photographs of red abalone lysin crystals grown at 295.3 K in 5 μl sitting drops containing 0.4 m*M* protein, 25 m*M* acetate buffer (pH 4.5, 5.3 or 5.5), 26.25 m*M* sodium citrate–citric acid, 0.35 m*M* boric acid (pH 4.5), 0.05 m*M* EDTA buffer (pH 4.5) and 0.1%(*w*/*v*) NaN_3 with the precipitants and additives as follows: (*a*) 0.500 *M* $(NH_4)_2SO_4$, (*b*) 0.615 *M* $(NH_4)_2SO_4$, (*c*) 0.425 *M* $(NH_4)_2SO_2$ and 10 m*M* NH_4SCN, (*d*) 0.400 *M* $(NH_4)_2SO_4$ and 10 m*M* NH_4SCN, (*e*) 30 m*M* NH_4SCN, (*f*) 0.600 *M* $(NH_4)_2SO_4$ and 0.01%(*w*/*v*) alcian blue 8GX, (*g*) 0.600 *M* $(NH_4)_2SO_4$ and 0.01%(*w*/*v*) pyridine derivative of alcian blue, and (*h*) 0.425 *M* $(NH_4)_2SO_4$ and 10 m*M* NH_4SCN.

(Fig. 1*g*). The organic additives dioxane, 2-methyl-2,4-pentanediol (MPD), glycerol and 1-propanol were effective solubilizing agents of lysin.

The largest single crystals of dimeric lysin were obtained after 3 weeks of growth at 295.3 K from a 5 μl sitting drop consisting of equal volumes of protein and reservoir solutions. The reservoir solution contained 750 m*M* $(NH_4)_2SO_4$ and 20 m*M* NH_4SCN as precipitants, 52.5 m*M* sodium citrate–citric acid–0.35 m*M* boric acid as precipitant and buffer (pH 4.5), 0.1 m*M* EDTA as additive and buffer (pH 4.5), 0.02%(*w*/*v*) OTGP as a growth promoter, and 0.1%(*w*/*v*) NaN_3 as preservative in glass-distilled water. The protein solution contained 14 mg lysin ml^{-1}, 0.1%(*w*/*v*) NaN_3, and a 50 m*M* acetate buffer (pH 5.5) in glass-distilled water. Maintenance of a 1 pH unit difference between protein and reservoir solutions appeared to minimize the growth of multi-crystalline forms (Fig. 1*h*) that usually resulted when these protein and reservoir solutions were buffered at the same pH.

Crystals grown at acid pH using ammonium sulfate as the precipitant contain a dimer in the asymmetric unit, have the space group $P222_1$ and unit-cell dimensions $a = 52.3$, $b = 47.0$, $c = 123.8$ Å with a Matthews (1968) coefficient of 2.4 $Å^3\ Da^{-1}$. Data to 2.7 Å resolution were 79% complete and were recorded with an MAR image plate. The R_{symm} (intensities) was 5.4 with an average redundancy of 3.2. The structure was solved by molecular replacement using the red lysin monomer as the search model. Refinement of the crystallographic molecular model is in progress.

In addition to existing as a dimer in the crystal structure, lysin from the red abalone was shown to exist as a dimer in solution at acid pH. Lysin oligomerization in solution was investigated using light-scattering techniques. An aliquot of the 14 mg ml^{-1} protein solution was diluted 100-fold with a 50 m*M* acetate buffer at pH 4.5, concentrated to 13 mg protein ml^{-1}, re-diluted tenfold with a 50 m*M* acetate buffer at pH 5.5, and re-concentrated to 14 mg ml^{-1}. Averaged dynamic light-scattering measurements of the aqueous protein preparations at both pH 4.5 and 5.5 indicated a 2.93 ± 0.06 nm hydrodynamic radius with an estimated molecular weight of 38.2 kDa (±20% with a 1.000 baseline). These results indicated that lysin from the red abalone is a dimer in solution at acid pH.

In summary, the anionic precipitants sulfate, acetate, phosphate and thiocyanate were effective in producing lysin crystals. Thiocyanate anions were much more effective than sulfate anions at precipitating the basic protein lysin as evidenced by the large crystals grown in sitting drops containing either 30 m*M* NH_4SCN or 0.6 *M* $(NH_4)_2SO_4$ as precipitant. The ability of thiocyanate and sulfate anions to function as co-precipitants was evidenced by the large crystals grown in sitting drops containing 0.4 *M* $(NH_4)_2SO_4$ and 10 m*M* NH_4SCN. Citrate, sulfate and thiocyanate anions were effective co-precipitants. Crystals were not obtained using carbonate or benzoate anions, although both were effective precipitants of lysin. This indicates that lysin possesses specificity for producing crystals *versus* precipitation depending on which anions are used for pairing. The quantitative effectiveness or competitive preference (relative to sulfate anions) of each anionic precipitant tested on lysin was determined from tested concentrations and ranked as follows: SCN^- (20×) > benzoate (10×) > CO_3^{2-} > citrate > CH_3COO^- ≃ phosphate (1.5×) > SO_4^{2-} (1×).

Additional crystal forms of red abalone lysin are being analyzed, and the results of this study are being used to design crystallization experiments for pink and black abalone lysins.

This work was supported by National Science Foundation grant MCB 9205020 (CDS) and National Institutes of Health grants HD 12986 (VDV), GM-38419 (EAS), GM-46192 (EAS) and AI-23498 (EAS). This is publication 8205-MB from The Scripps Research Institute.

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