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A new additive for protein crystallization

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Abstract The potential usefulness of the new zwitterionic solubilizing agent, dimethyl ethylammonium propane sulfonate (NDSB195), in protein crystallization was shown using hen egg-white lysozyme. In the presence of this agent, highly diffracting crystals were obtained using ammonium sulphate as a precipitant, whereas in its absence only amorphous precipitates were obtained. The crystals possess a triclinic unit cell not previously described and diffract to a resolution of 2 Å. To ascertain that the new reagent had not produced significant changes in the protein fold the structure was determined to a resolution of 2.6 Å. Only minor differences were observed (notably in regions of crystal contacts) with the known tetragonal lysozyme structure (Brookhaven Protein Data Bank entry 1HEL).

Key words: Crystallization; Solubility

1. Introduction

Protein crystallization occurs by a process of precipitation from super-saturated protein solutions. These are most frequently obtained by the addition of a precipitation agent often coupled with the reduction of the volume of the solution. The exact conditions for obtaining crystals may be difficult to define, a most common result of increasing precipitant concentration being the production of an amorphous precipitate. The solvent factors involved in determining whether crystals or amorphous precipitate are obtained remain a big unknown. Various studies by dynamic light scattering have shown that crystal nucleation is generally characterised by a relatively sharp transition from a monodisperse solution to insolubility as the precipitant concentration is increased, whereas a continuum of aggregates before precipitation leads to amorphous material [1–3].

The hypothesis that we explore in this paper is that the addition of a non-detergent protein solubilizing (non-denaturing) agent to a protein crystallization mixture might prevent the formation of amorphous aggregation at the incipient precipitation condition and promote crystallization. To this end we have investigated the synergistic effect of dimethyl ethylammonium propane sulfonate, NDSB195, a new zwitterionic solubilizing and stabilising agent developed in our laboratory, on the crystallization of hen egg-white lysozyme with ammonium sulphate. We have chosen this protein, firstly because it is often used in model protein crystallization studies, but principally because it is notorious for producing amorphous or gel-like precipitates and not crystals with the popular salting-out agent ammonium sulphate [1,4]. In particular this protein has been used in one dynamic light scattering study to illustrate the differences under precrystallisation conditions between the effects of sodium chloride which induces crystal formation and ammonium sulphate which does not [1,4]. Although this report is not intended to be a crystallographic analysis of a new crystal form of lysozyme, which was obtained in this study, we felt it obligatory to verify that the protein fold in this crystal form does not differ significantly from that already established by crystallization in the presence of other salts.

2. Materials and methods

2.1. Reagents

Propane sultone was from Fluka, and mixed ion-exchange beads (Type TMD 8) were from Sigma. Lysozyme was obtained from Boehringer. All other chemicals (analytical grade) were obtained from Merck.

2.2. NDSB synthesis

NDSB195 (dimethyl ethylammonio propane sulphonate) was synthesised as follows. A 3-necked round-bottomed flask, equipped with reflux condenser and thermometer, was set in a beaker filled with water at 20°C. In this was placed 100 g propane sultone (0.83 mol) dissolved in 500 ml dichloroethane. Dimethyl ethylamine (0.9 mol) was added slowly to the moderately stirred solution. After overnight reaction at room temperature, the product precipitated. After crystallization from methanol/water mixtures (typically 98:2 w/w) and redissolving in water, NDSB195 was purified on a mixed-bed ion-exchange resin. The final product was characterised by NMR. It should be noted that propane sultone is carcinogenic and should be handled with care.

2.3. Crystallization

Crystallization assays were performed in silicone-coated multi-vial plates using a batch technique. Hen egg-white lysozyme was dissolved to 15% in 50 mM sodium acetate, pH 4.6, in the presence or absence of 1 M NDSB195, and mixed 1:1 with various salt solutions as described below.

2.4. X-Ray diffraction experiments

Data were collected to 2.6 Å resolution on a rotating anode generator operated at 40 kV/100 mA equipped with a 18 cm MarResearch image plate detector. 180° of data were collected in 2.5° frames on a single crystal rotating about an arbitrary axis. Unit cell parameters and space-group (P1) were determined initially by the auto-indexing routines of the XDS programme [5] and confirmed by subsequent analysis. The structure was solved by molecular replacement using the known triclinic lysozyme structure (2LZT in the Brookhaven Protein Data Bank, [6]) with the programme AMORE [7]. Refinement was performed with XPLOR [8] using standard protocols of energy minimisation and B-factor refinement, after manual adjustment of some loops and residues involved in crystal contacts with FRODO [9].

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Abbreviations: NDSB, non-detergent sulfobetaine; NDSB195, dimethyl ethylammonium propane sulfonate.

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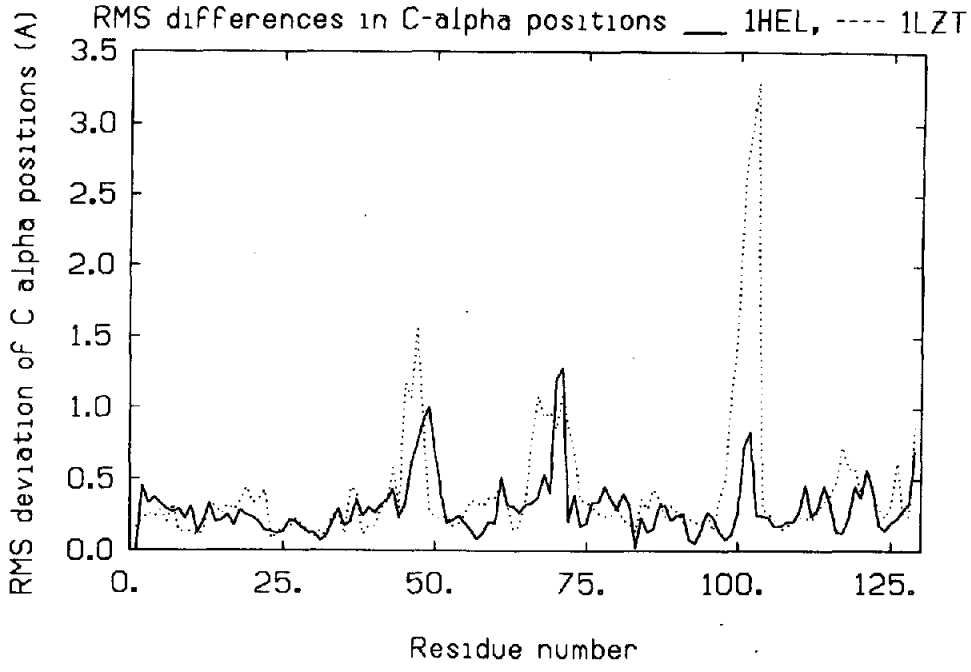

Fig. 1. Root-mean-square (RMS) differences in Cα positions of one of the lysozyme molecules in the new crystal form compared to other lysozyme structures (1HEL, tetragonal lysozyme; 2LZT, trigonal lysozyme).

3. Results and discussion

The initial, control, tests of growing crystals of lysozyme by established procedures from sodium chloride solutions at pH 4.6 showed that crystallization was significantly different in the presence of NDSB195. It was found that crystallization could be carried out with protein solutions at concentrations as high as 75 mg/ml and that the size of the crystals produced correlated with the concentration of NDSB195. At this protein concentration in the absence of NDSB195 showers of micro-crystals were obtained whereas in the presence of NDSB195 at concentrations from 0.5–1 M large (up to $0.5 \times 0.5 \times 0.75$ mm), well-diffracting (at least 2 Å resolution), tetragonal crystals were obtained. Furthermore the growth rate of the latter was much higher in the presence of NDSB195 where crystals of average dimensions $0.1 \times 0.1 \times 0.1$ mm could be obtained in less than 1 h.

Although there has been a recent report of the crystallization of hen egg-white lysozyme in the presence of sulphate ions [10], this was achieved at low concentrations of sulphate at pH values much higher than those used in the presence of a variety of other salts. At pH 4.6, addition of ammonium sulphate (over 18% saturation) to lysozyme solutions resulted in precipitation and even in the formation of gels, as previously reported [1,4]. In the presence of 0.5 M NDSB195, with ammonium sulphate solutions at 22% saturation, crystals formed over a few days. The crystals were rhomboidal prisms with typical dimensions of $0.1 \times 0.1 \times 0.2$ mm. Although the crystals had a strong tendency to grow in clusters it was possible to separate single crystals for X-ray diffraction analysis.

The crystals belong to space-group P1 with cell dimensions $a = 41.7$ Å, $b = 58.4$ Å, $c = 58.9$ Å, $\alpha = 116.5°$, $\beta = 97.6°$, $\gamma = 105.7°$. The diffraction limit on the conventional X-ray source was about 2 Å. A dataset at 2.6 Å resolution was collected and the structure solved by molecular replacement using the published structure of triclinic lysozyme (entry 2LZT in the PDB). The root main square deviations from ideal geometry are 0.014 Å for bond length and 2.95° for bond angles (using the XPLOR19X parameter set). Each of the three independent molecules in the asymmetric unit shows a similar conformation. The conformation of the loop formed by residues 99–103 is close to that found in tetragonal lysozyme (PDB entry 1HEL [11]) rather than that in triclinic lysozyme (see Fig. 1). Other significant differences in Cα positions for residues 45–48 and 67–71, both sequences previously noted as flexible regions [6,11,12]. The side chain of Trp^{62}, a residue implicated in substrate binding, is displaced due to close crystal contacts, and this in turn causes a large displacement of Arg^{73}. Several clear water molecules are visible in the current difference map, as well as a few large positive peaks which could be sulphate ions. One large extra density, not in a region of positive charge, could be a partially ordered NDSB195 molecule.

At present, and in the absence of further physicochemical data, we do not have a clear understanding of the effects of NDSB on proteins. Since NDSB is a solubilizing agent (Vuillard et al., manuscript in preparation) a possible explanation of the results reported in this present work is that NDSB acts by preventing amorphous aggregation. This hypothesis would be in agreement with work linking the ability to crystallize with the existence of a sharp transition from a monodisperse solution to crystals when the concentration of precipitant is increased [1–3]. This hypothesis fits well with both observations of crystallization of lysozyme with sodium chloride and ammonium sulphate in the presence of NDSB195. This argument will be pursued further in the study of other proteins which have proved to be intractable in previous crystallization studies.

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