Acta Cryst. (1994). D**50**, 396–397

Investigation of Nucleating Lysozyme Solutions

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(*Received* 29 *October* 1993; *accepted* 30 *November* 1993)

Abstract

This laboratory has explored the potential of a combination of three analytical techniques to study the nucleation of chicken egg-white lysozyme. Collisional quenching of the fluorescent molecule SPQ [6-methoxy-*N*-(3-sulfopropyl)quinolinium] by chloride ions was used to determine the binding of the crystallizing agent to the protein at equilibrium and kinetically. Calorimetric measurements show that this binding generates an exothermic peak larger than the energy released during the early stages of nucleation. Light scattering intensity measurements were used to follow the aggregation kinetics.

Introduction

While the technology exists today to develop sophisticated hardware to crystallize proteins in microgravity, important knowledge of the structure of the crystallizing medium remains to be gained. With this approach in mind, our laboratory has combined microcalorimetry, light scattering intensity measurements and a fluorescence quenching technique to study the interactions between protein and crystallizing agent and their role in the nucleation process. Our choice of chicken egg-white lysozyme was dictated by the abundance of this protein and the extensive solubility data gathered by co-workers (Cacioppo, Munson & Pusey, 1991). Among various crystallizing conditions, we chose to examine lysozyme in its tetragonal form at pH 4.0 in 0.1 *M* sodium acetate (NaAc) buffer using NaCl as crystallizing agent (Alderton & Fevold, 1946). The following summarizes the results obtained to date and fuller reports of our findings are in preparation.

Results and discussion

The molecule SPQ fluoresces at 440 nm when excited by light at 350 nm. In the presence of anions such as Br^-, Cl^- or I^-, its fluorescence is quenched in a purely collisional way (Wolfbeis & Urbano, 1982, 1983). The intensity ratio of the unquenched signal F_o over the quenched signal F is a direct measure of the concentration of the quenching anion Q according to the Stern–Volmer equation,

$$F_o/F = 1 + K_q[Q].$$

The quenching constant K_{Cl} for chloride ions was determined experimentally in 0.1 *M* NaAc buffer at pH 4.0 ($K_{Cl} = 92\ M^{-1}$ in buffer and $K_{Cl} = 117\ M^{-1}$ in deionized water). Lysozyme molecules in solution were found to be significant collisional quenchers of SPQ and obeyed the Stern–Volmer relation. Although it was possible to account for the effect of multiple collisional quenchers such as simple anions, we found that this technique could not be used effectively in a titration type experiment to quantitate the binding of chloride ions to lysozyme. Using a dialysis method, we were able to follow the binding process at equilibrium by determination of the free Cl^- concentration in the lysozyme-free dialysed solution. Tanford & Wagner (1954) determined that 13 Cl^- were bound to lysozyme at pH 4 at 0.15 *M* KCl and 5.5 Cl^- at 1.0 *M* KCl based on a potentiometric technique at 298 K. These numbers agree well with our assay in spite of our use of NaCl and at 293 K. The rapid response of the molecular probe coupled with its stability allowed us to explore the range of conditions within Tanford's bracket. The collected data display several interesting features: from 0 to 0.35 *M* NaCl the number of Cl^- ions bound increases steadily to a peak value of 21–23 ions per lysozyme molecule. The number of bound ions remains constant within experimental error between 0.35 and 0.6 *M* NaCl, after which it decreases sharply to the value of about nine anions per lysozyme at 0.8 *M* NaCl. The phase diagram of tetragonal chicken egg-white lysozyme shows that the limit of solubility is 24.5 mg ml^{-1} at 293 K and pH 4.0 when the concentration of NaCl reaches 2% or 0.34 *M* (Cacioppo & Pusey, 1991). Our data show that the desolubilization of the protein coincides with the saturation of the possible Cl^- binding sites. As we increase the concentration of NaCl, nucleation occurs and crystallization takes place while we observe fewer Cl^- ions bound to the protein. It is interesting to note that only one Cl^- has been crystallographically located in tetragonal hen egg-white lysozyme (Blake *et al.*, 1967), although none

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ISSN 0907-4449 © 1994

appear in the Protein Data Bank at Brookhaven National Laboratory (Bernstein *et al.*, 1977; Abola, Bernstein, Bryant, Koetzle & Weng, 1987).

The kinetic study of Cl^- binding to lysozyme was performed with a pneumatic stopped-flow apparatus capable of recording data with a dead time of 2 ms. Data were collected on both undersaturated and supersaturated lysozyme solutions as we mixed salt-free lysozyme and NaCl solutions. We followed the evolution of the SPQ fluorescence and light scattering intensity on identical solutions to confirm the existence and the rate of protein aggregation. Even under dilute concentrations of NaCl, we were unable to detect the fast rate of binding of Cl^- to the protein, which would have been observed as a rapid increase of the fluorescence signal. Thus the binding reaction had gone to completion within the 2 ms dead time of the instrument. This event was followed by the slow decay of the SPQ fluorescence signal indicating a subsequent release of bound Cl^-. Stopped-flow light scattering intensity measurements confirmed the formation of aggregates over the same time period.

Calorimetric measurements were performed with a stopped-flow conduction type microcalorimeter designed and built in part in house. Enthalpies of ion binding on lysozyme were obtained for several lysozyme and chloride concentrations. Due to the large exothermic exchange attributed to anion binding, measurements of the subsequent heat of aggregation proved to be difficult to obtain. Although the heat of binding per anion is comparable to the heat of protein–protein association per lysozyme molecule (Ross & Subramanian, 1981), the number of ions involved in the first reaction is much greater than the number of macromolecules in the second. This results in a larger amount of heat released in the case of Cl binding. The calorimetry data confirm the rapid binding of the ions by exhibiting a large exothermic peak with a slope of the order of 200 to 1000 J s^{-1} within the first second depending upon the salt concentration. We found that the enthalpy of binding per mole of bound Cl^-, ΔH_{Cl}, was of the order of -150 to -190 J mol^{-1} of lysozyme. Upon integration of the recorded heat over time, we found that a slowly evolving exothermic reaction takes place long after the initial ion-binding event. The recorded signal cannot be attributed to any drift in the instrument baseline since we measured the former at less than 1%. The aggregation of lysozyme molecules is believed to be the cause for this observation.

This work was supported in part by the Universities Space Research Association under contract NAS8-38785. We also wish to thank the Centre National d'Etudes Spatiales in Toulouse, France, for financial support of LS in the early stages of the project.

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