# A Two-State Conformational Transition of the Extracellular Ribonuclease of *Bacillus amyloliquefaciens* (Barnase) Induced by Sodium Dodecyl Sulfate†

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ABSTRACT: Barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens,* is shown to undergo a reversible two-state conformational transition at 0.65 m*M* sodium dodecyl sulfate (SDS) at 37°. The principal evidence is based on the equivalence of two independent values of the SDS–barnase binding ratio; about 14 mol of SDS/mol of barnase. Both were derived from fluorometric titration data, one being based on simple conservation of SDS and the other on the use of Wyman's theory of linked functions. No SDS is bound to barnase at SDS concentrations below the transition region.

Studies of protein–detergent interaction are of increasing interest because of their application to theories of biological membrane structure and to the problems of sequence-determined folding and stability of proteins.

There is evidence (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970) that, at high sodium dodecyl sulfate (SDS)[1] concentrations, all protein–SDS complexes have the same basic structure if all disulfide bridges or other cross-links have been broken. Thus, disulfide bonds are often reduced in order to study the complex. When this is done, however, it is not possible to study the equilibrium between the native protein and its SDS-altered form. On the other hand, if the cross-bridges remain intact, specific constraints are put on the conformation of the complex.

Barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens,* is composed of a single chain of 110 amino acids of known sequence (Hartley and Barker, 1972). It has no half-cystine residues and no metal or other nonpeptide moieties. It can be reversibly unfolded by a variety of denaturing agents (Hartley, 1968). It serves, therefore, as a model by which the reversible transition between a native protein molecule and its sequence independent SDS–protein complex can be studied. In this communication a study of the cooperativity and stoichiometry of such a transition of barnase is reported.

## Materials and Methods

Barnase was prepared as described earlier (Hartley and Rogerson, 1972). Sodium dodecyl sulfate was the "specially pure" grade of BDH Chemicals, Ltd. Solutions were never more than a week old. A single batch of $^{35}S$-labeled SDS was obtained from New England Nuclear. Appropriate dilutions into buffer or cold SDS solutions were made from concentrated stock solution of this material. Verifiable SDS in the stock solution declined rapidly with a half-life of several weeks, presumably due to radiolysis. Only experiments performed within the first few days and which require only the assumption that a substantial fraction of the label is SDS are reported.

A Perkin-Elmer Hitachi, MPF-3L spectrofluorometer was used for fluorescence spectra and for fluorometric titrations. Absorption spectra and titrations were done with a Beckman Acta III spectrophotometer. Both instruments included jacketed cuvet holders and temperature was determined by use of a thermistor in a dummy cuvet alongside the sample. Samples for titration were sealed into standard 1.0 cm × 1.0 cm cuvets by cementing a square of ⅛ in. thick silicone rubber across the top with General Electric RTV-102 silicone rubber sealant. Titrant from a syringe microburet was introduced by means of a fine (1.09 mm o.d.) polyethylene tube pressure-fitted into a smaller hole bored through the silicone rubber cap. The cuvet could be inverted to mix after each addition of titrant without leakage.

SDS assays were performed essentially as described by Reynolds and Tanford (1970a). The sample (0.02 or 0.05 ml) was added to a tube containing 10 ml of chloroform and 2.5 ml of a 25 mg/l. solution of Methylene Blue. After shaking and separation, absorption of the bottom phase was read at 655 nm and compared to a standard calibration curve. $^{35}S$ was counted in a Beckman LS-250 liquid scintillation counter using "Hydromix" cocktail from Yorktown Research Co., Yorktown, N.Y.

Barnase, as protein, was assayed by measuring absorption at 280 nm using $A_{280}$ (1 mg/ml) = 2.09. When SDS was present the sample was diluted to reduce the SDS concentration to below 0.4 m*M* and the barnase was allowed to recover its native spectrum before reading. Assay for ribonuclease activity has been described (Rushizky et al., 1963).

A simple two-chambered device was used for equilibrium dialysis. The volume of the sample chamber was about 1.0 ml, that of the dialysate about 2.0 ml. The two chambers could be changed or sampled independently. The membrane used was Spectropor 3 (Spectrum Medical Industries, Los Angeles, Calif.). The membrane was washed by soaking in many changes of SDS-containing buffer to remove material absorbing at 280 nm. During experiments a small correction was made for the amount of such material that still remained by reading the absorption of the dialysate as well as

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[1] Abbreviation used is: SDS, sodium dodecyl sulfate.

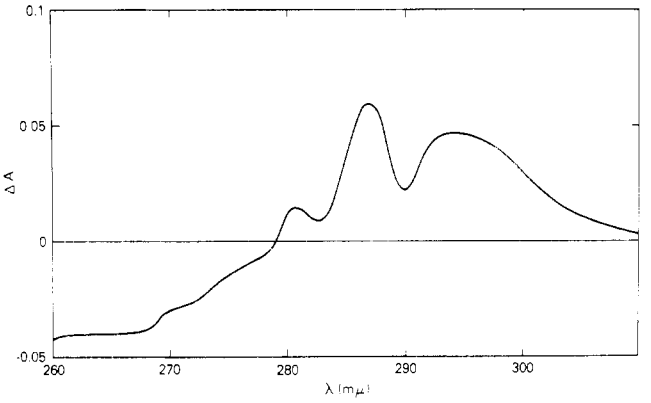


FIGURE 1: Difference spectrum. Native barnase minus barnase in 8.5 m$M$ (total) SDS. Both 0.05 $M$ $NH_4HCO_3$ (pH 8), 37°; 0.23 mg/ml of barnase ($A_{280}$ (native) = 0.506).

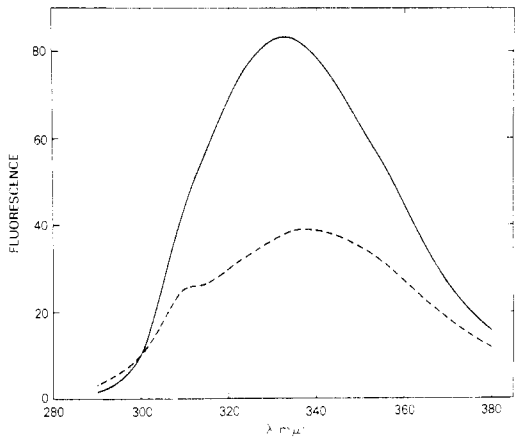


FIGURE 2: Uncorrected fluorescence spectra of native and SDS-barnase. (—) 9.5 μg/ml of barnase in 0.05 $M$ $NH_4HCO_3$ (pH 8), 37°; (- - - -) same plus SDS to 5.5 m$M$. Excitation wavelength 280 nm. Shoulder on SDS spectrum is due to Raman scattering of solvent. Ordinate is arbitrary.

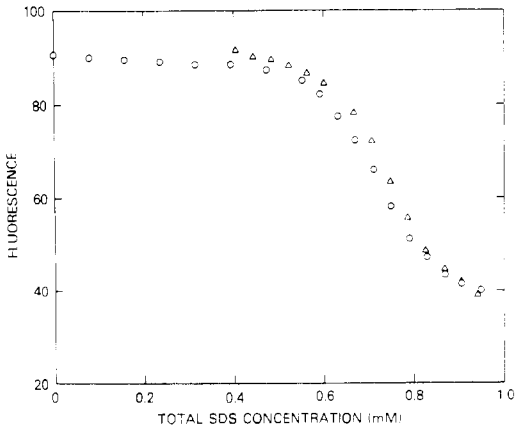


FIGURE 3: Reversibility of the transition. (○) First titration; 23 μg/ml of barnase in 0.05 $M$ $NH_4HCO_3$ (pH 8), 37°. Titrant 0.346 $M$ SDS. (△) Second titration, same sample diluted to 10 μg/ml of barnase with same SDS-free buffer. Excitation at 279 nm, emission at 330 nm. The ordinate is arbitrary, the instrument sensitivity being adjusted to give about the same reading at the start of each titration.

that of the sample. Equilibrium with respect to SDS was reached within 6–8 hr with gentle shaking at 37°. For the experiments reported, 24–48 hr was allowed.

At SDS concentrations near that just necessary to induce the cooperative structural transition in barnase, the rate of

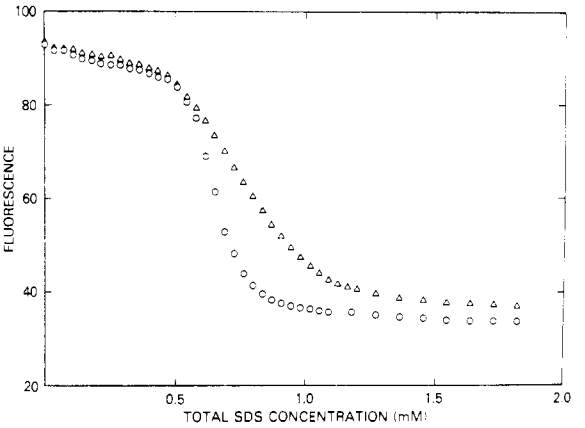


FIGURE 4: Titration of different barnase concentrations. (○) 0.0241 mg/ml, (△) 0.331 mg/ml. Both in 0.05 $M$ $NH_4HCO_3$ (pH 8), 37°. Titrant 0.346 $M$ SDS. Excitation at 279 nm, emission at 330 nm. Ordinates arbitrary, as Figure 3.

the reaction is highly temperature dependent. At 25°, the time necessary to reach equilibrium ranges from hours to days and titration experiments are not practical. At 37°, the temperature chosen for these experiments, equilibrium was generally reached within 5 min and this temperature is also sufficiently below 50°, near which barnase undergoes a structural transition in the absence of SDS (Hartley, 1968).

At high barnase concentrations, a precipitate forms as SDS is added above about 0.5 m$M$, dissolving at higher SDS concentration. The barnase concentration below which solubility is not a problem is a function of pH, ionic strength, and the particular salts and buffers used. It was this characteristic which dictated the choice of buffer (0.05 $M$ ammonium bicarbonate, pH 8.0) used in these experiments. Also, at this ionic strength the critical micelle concentration of SDS is about 2 m$M$ (Emerson and Holzer, 1967), so that free SDS concentration is not limited by micelle formation in the transition range.

## Results

Figures 1 and 2 show the spectral changes of barnase that take place in excess SDS. The absorption difference spectrum is qualitatively similar to that seen in thermal denaturation (Hartley, 1968) and indicates increased access of solvent to tyrosine and tryptophan side chains. The change in absorption is only about half as great as in thermal denaturation, however. The decrease in fluorescence, due mainly to changes in the environment of tryptophan chromophores, is much greater. As the fluorescence change at 330 nm could be measured over a much wider range of barnase concentration, it was used as the criterion of barnase unfolding in the principal experiments reported here. The excitation wavelength, 279 nm, was chosen to minimize the effects of absorption changes through the transition.

Figure 3 demonstrates the reversibility of the transition as observed by fluorescence. The two titrations were done on the same sample, with a simple dilution in SDS-free buffer and an increase in instrument sensitivity intervening.

In all the experiments reported here, ribonuclease activity was fully recovered after the SDS concentration was sufficiently reduced by dilution. There is some ambiguity in these results because a low SDS concentration in the assay increases the apparent activity by as much as 20%, presumably due to its effect on the RNA substrate. This is true up to about 0.5 m$M$ SDS, above which the activity drops off

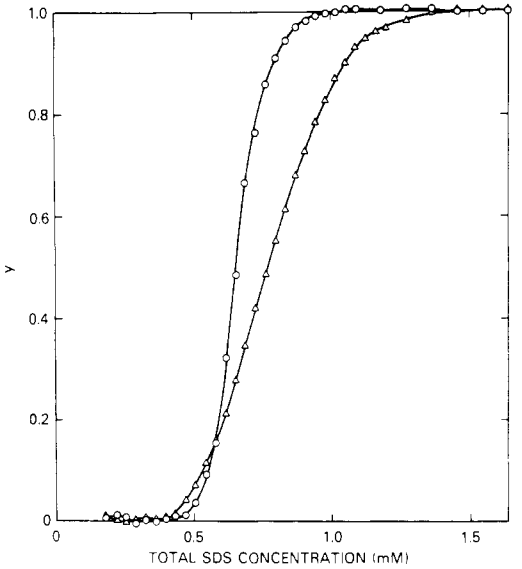


FIGURE 5: Titrations of Figure 4, with ordinates expressed as $y$, the fraction of the total fluorescence change. Corrections have been made for the more gradual fluorescence changes outside the transition region. Symbols as Figure 4.

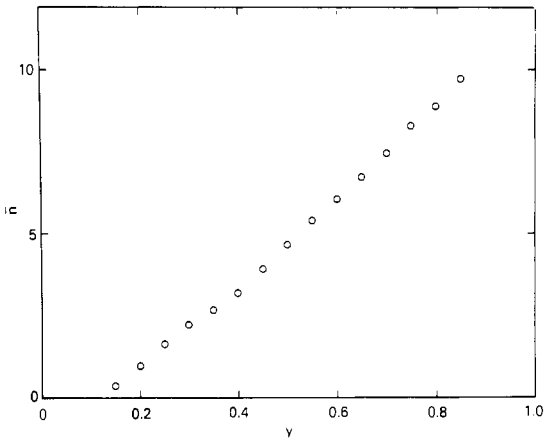


FIGURE 6: $\bar{n}$, the average number of SDS molecules bound per molecule of barnase. Derived from Figure 5 as described in the text.

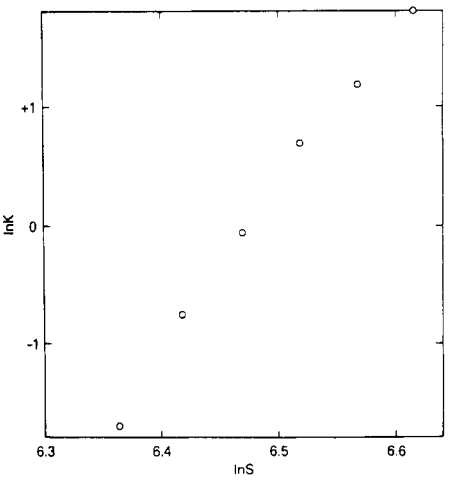


FIGURE 7: Ln $K$ vs. ln $S$. $K$, the equilibrium constant, and $S$, the free SDS concentration, derived from data of Figure 4 as described in text.

rapidly. The precipitate formed in the transition region at high barnase concentration is similar to that formed on thermal denaturation (Hartley, 1968) in that it does not dissolve on dilution of the SDS as the thermal precipitate does not on cooling. Both precipitates, however, are readily dissolved by excess SDS and can be recovered in solution and fully active on subsequent dilution of both the protein and the SDS.

In Figure 4 are shown the results of titrating two widely different concentrations of barnase with SDS. The fact that both transitions start at about 0.45 m$M$ SDS suggests that few, if any, SDS molecules are bound to native barnase, as each such molecule bound to a barnase molecule at 0.027 m$M$ barnase concentration would reduce the free SDS concentration by 0.027 m$M$. As the transition proceeds, however, it is clear that substantial amounts of SDS are being removed from free solution by the higher barnase concentration, thereby increasing the total SDS necessary to complete the transition. These observations can be quantified in the following manner. In Figure 5 is seen the data of Figure 4 expressed as the fraction, $y$, of the total fluorescence change for each titration. Corrections have been made for the change of fluorescence with SDS outside the transition range. If $y$ is a function only of free SDS concentration, the amount of SDS bound for each value of $y$ is a simple function of the difference in total SDS concentration between the two curves:

$$\bar{n} = ([S_2'] - [S_1'])/([B_2] - [B_1])$$

where $[B_1]$ and $[B_2]$ are the molar barnase concentrations in the two experiments, $[S_1']$ and $[S_2']$ are the total molar SDS concentrations for a given value of $y$ and $\bar{n}$ is the average number of SDS molecules bound per barnase molecule. Figure 6 is a plot of $\bar{n}$ vs. $y$ extracted from the data of Figure 5. While the values of $\bar{n}$ derived by this treatment do not depend on the assumption of a two-state transition, the straight line obtained is consistent with such a transition with 13.6 ± 1 extra SDS molecules bound to each unfolded barnase molecule, this number being nearly constant over the fairly narrow range of the transition. Since values of $\bar{n}$ much less than zero cannot be real, the extrapolated values, $\bar{n} = -2.1$ at $y = 0$ and $\bar{n} = 11.5$ at $y = 1$ are both probably low due to some systematic error. In any case, the differences between these values and 0 and 13.6, respectively, are not great enough to affect the general argument of this communication.

Wyman (1964) (and see Tanford, 1970) has shown that for a strictly two-state transition, the difference in the number of denaturant molecules bound to the protein in the two states is given by:

$$\Delta n = d(\ln K)/d(\ln [S])$$

where $K$ is the equilibrium constant for the transition and [S] is the free SDS concentration. For the lower barnase curve of Figure 5, $[S] = [S'] - \bar{n}[B]$ has been calculated. This involves only a small correction to [S'] since $\bar{n}[B] \ll [S']$. The equilibrium constant is given by $K = y/(1 - y)$. Thus we can plot (Figure 7) ln $K$ vs. ln [S]. The straight line obtained yields $\Delta n = 14.0 \pm 1$, in agreement with the independent calculation based simply on conservation of SDS. The center of the transition, where $K = 1$, is at 0.65 m$M$ SDS.

In another titration experiment, the absorption at 286 nm of a 3.0-mg/ml sample of barnase was followed as well as the fluorescence at 330 nm. The center of the transition, as observed by both parameters, was 0.69 m$M$ total SDS.

Table I

| SDS Concn (m*M*) | Barnase Concn (m*M*) | SDS/Barnase Molar Binding Ratio |
|---|---|---|
| 0.18 | 0.151 | 0.1 |
| 1.56 | 0.222 | 17.0 |
| 1.72 | 0.390 | 22.5 |
| 1.92 | 0.379 | 35.1 |
| 2.10 | 0.172 | 40.6 |

Table I shows the results of several equilibrium dialysis experiments. Reliable measurements in or near the transition region were not possible due to solubility problems. The results shown are compatible with those obtained by fluorometric titration, but it is shown that binding to the unfolded form increases considerably above the transition range. An attempt at quantitative binding studies at low barnase concentration was made by chromatography on G-25 Sephadex equilibrated with $^{35}$S-labeled SDS. In general, quantitatively useful data were not obtained due to the rapid degeneration of the labeled SDS. Two types of experiment, however, did yield definitive results, demonstrating that barnase does not bind any SDS at concentrations below the transition zone. Sample and column were both at 37° in the following two experiments. A 0.8-ml sample of barnase, 1.0 mg/ml in 1.5 m*M* SDS (including label), was passed through a 12-ml column equilibrated with 0.4 m*M* SDS with an activity of 320,000 cpm/ml. No extra counts were associated with the emerging barnase peak. Under these conditions 0.05 mol/mol or more bound SDS could have been detected. In another experiment a similarly labeled sample of barnase in SDS was put through a column equilibrated with buffer only. No radioactivity was associated with the barnase peak. In this experiment 0.005 mol/mol could have been detected. Thus, no SDS remains bound to barnase below the transition range, even after exposure to high SDS concentrations.

## Discussion

The value of $\Delta n$ calculated from the shape of the titration curve using Wyman's theory of linked functions is a minimum value. Only if the transition is essentially two-state will it represent the true binding value. If an appreciable fraction of intermediate or alternate molecular forms of barnase coexist with the native and SDS forms in the transition range, the true value of $\Delta n$ must be greater. The value obtained from the difference between the curves for two barnase concentrations, on the other hand, does not depend on the two-state hypothesis. The equivalence of the two numbers, therefore, is theoretically rigorous evidence for an all-or-none transition. This conclusion is analogous to that reached when the calorometrically determined $\Delta H$ of a thermal transition agrees with the value of $\Delta H$ from a van't Hoff plot (e.g., Tsong et al., 1970).

The equivalence of the SDS "melting" concentration observed by absorbance at 286 nm and fluorescence at 330 nm is further evidence for a two-state transition since these parameters depend on changes in the environment of different chromophores.

It is not implied that transient intermediate states do not exist. Preliminary kinetic studies (at 34°) have readily shown that at least two steps are involved in the transition, with rates differing by a factor of 2. Dissection of such stepwise reactions, using observational parameters depending on different aspects of structure (circular dichroism, absorption, fluorescence, etc.), should be useful in exploring the sequence of events in the folding and unfolding of this model protein.

There is agreement between three different techniques, fluorometric titration, gel filtration chromatography, and equilibrium dialysis, all indicating that no SDS is bound to barnase below the transition region. This should simplify theoretical interpretation of further SDS–barnase experiments. For many but not all (Pitt-Rivers and Impiombato, 1968) proteins, the complete removal of SDS is very difficult (e.g. Weber and Kuter, 1971). The present example confirms that tight binding of a small number of SDS molecules is not a general property of proteins.

The binding of 14 molecules of SDS to one of barnase in the transition region is equivalent to 0.32 g/g. This is reasonably close to the value of 0.4 g/g found by Reynolds and Tanford (1970a) for a number of proteins at 0.5 m*M* SDS. The somewhat lower value and the slightly higher SDS concentration required may be specific for barnase or may be due to the higher temperature (37° vs. 25°) used in these experiments. The increased binding at high SDS concentration, with little further spectral change, is also in agreement with their results, except that the increase appears to be more gradual in this case.

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