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Some Environmental Effects on the Thermal Transition of *Bacillus amyloliquefaciens* Ribonuclease (Barnase)*

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ABSTRACT: The thermal transition temperature, T_m, of barnase is unaffected by pH in the range 5–9, but decreases outside of this range.

Below pH 5 the T_m closely parallels that of pancreatic ribonuclease and, as with that enzyme, the decrease in T_m at low pH is sharply reduced by sulfate. The T_m is not markedly affected by phosphate at any pH. The increase in proton binding which accompanies unfolding has a maximum of 3 or 4 at about pH 3.5, suggesting a similar number of abnormal carboxyl groups. Only two of the six tyrosines are ionized at pH 12.2 in the folded form. Above pH 7, stability is reduced by 10^{-6} M Cu^{2+}; 10^{-5} M guanosine monophosphate has little effect on the T_m but reduces the solubility of the unfolded form.

The general nature of the thermal transition of barnase has been documented in a previous paper (Hartley, 1968). It was shown that the reaction could be considered, to good approximation, to progress as a single step from the native folded form to a completely disorganized random coil. The reaction is reversible and the two forms are in equilibrium with one another in the transition range. Since barnase has no covalent cross-links (Lees and Hartley, 1966), it follows that the amino acid sequence fully determines the folded structure of the active enzyme. Studies of this transition in various environments and with various chemical and genetic modifications of the enzyme should yield clues as to how the different residues contribute to the form and stability of the folded molecule.

In this paper are reported the effects of various reagents on the transition of the unmodified, wild-type enzyme, with particular reference to the hydrogen ion concentration.

Materials and Methods

Our procedure for the production and purification of barnase has undergone more or less continuous modification since last reported (Lees and Hartley, 1966) and an up-to-date description is in preparation. The product, however, is similar, perhaps slightly purer, and certainly more reliably free of contaminating proteases. The last point is of utmost importance for the type of experiments reported here (and in Hartley, 1968). The enzyme in its folded form is quite stable in the presence of such proteases, but as the transition temperature is approached (in a variety of solvents) the unfolding reaction is rapidly driven to completion by the digestion of the unfolded form.

Methods for obtaining spectra and for spectrophotometric and gel filtration observations of the transition, etc., were reported previously (Hartley, 1968).

The pH values reported were measured with a Corning Model 12 pH meter. Those of the solutions used in the spectrophotometric transition experiments were determined directly after the experiments.

Results

Figure 1 shows the pH dependence of the transition temperature below pH 8. These data were all determined with the ionic strength ($\Gamma/2$) at 0.1, except at pH 0.7 (0.2 N HCl), and the two points for which NH_4Cl was added to make $\Gamma/2 = 0.2$. The points marked by open circles represent runs in which no buffer was included, the salt being NH_4Cl. For the other points, various buffers were used as noted in the figure legend. That some care must be taken in the choice of buffers may be seen. The transition temperature was depressed by the excess of acetic acid below its pK and, similarly, by basic imidazole above its pK. The same effect occurs with Tris in the upper end of its buffering range, but formic acid appears to be innocuous.

Below pH 0.7, reliable transition temperatures are difficult to obtain due to the decrease in solubility of the unfolded

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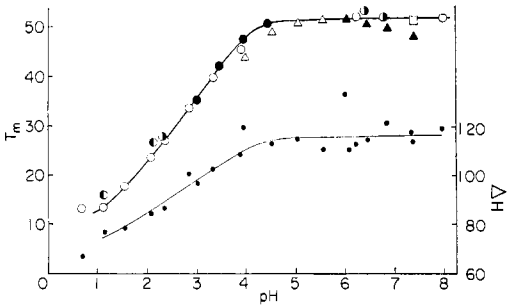

FIGURE 1: T_m and ΔH as a function of pH in the acid range. T_m: left-hand scale, heavy line, and points: (○) $\Gamma/2 = 0.1$ ammonium chloride, (◐) $\Gamma/2 = 0.2$ ammonium chloride, (●) $\Gamma/2 = 0.1$ ammonium formate, (◑) $\Gamma/2 = 0.1$ ammonium phosphate, (△) $\Gamma/2 = 0.1$ ammonium acetate, (▲) $\Gamma/2 = 0.1$ imidazolium chloride, and (□) $\Gamma/2 = 0.1$ Tris chloride. ΔH: right-hand scale, light line, and points (•).

form (concomitant with increasing ionic strength at any pH). The folded form, however, is highly soluble even at pH 0.5 and an estimate of the T_m can be obtained by observing the temperature at which turbidity first appears. Experience suggests that this temperature is some 3–6° below the actual T_m. At pH 0.5 (0.32 N HCl) turbidity appears only above about 12.5°, suggesting that the T_m is indeed leveling off below pH 1. It is difficult to unscramble the effects of pH and ionic strength in this range, however, and this conclusion must be tentative.

In Figure 1 is also shown the effect of pH on the enthalpy of the transition as calculated from van't Hoff plots of the data (see Hartley, 1968). For only a few of the experiments was enough data taken to allow correction for the change in OD_{286} outside the transition range, so the results shown in Figure 1 were calculated without this base line correction. The error introduced thereby in T_m is negligible (<0.5°) but the ΔH values are underestimated by about 15%. Thus the actual value of ΔH in the neutral region is about 140 kcal.

The pH dependence of a two-state transition of this nature must be due to the preferential binding of protons to the form favored by low pH. The excess of protons bound to the unfolded molecule over the native form at any pH is given by $\Delta r = \partial(\log K)/\partial pH$ (Hermans and Scheraga, 1961b), where K is the equilibrium constant. Δr has been calculated from the data as a function of pH essentially as described by Hermans and Scheraga (1961b), using the temperature at which $K = 1$. The results are shown in Figure 2.

In Figure 3 may be seen the effects of sulfate on the transition in the acid range. The line is that of Figure 1, showing the T_m without sulfate. The points are the values obtained in the presence of 5 mM sulfate. It should be noted that at pH 1.1 the concentration of divalent sulfate is only 0.65 mM while its effect is as great or greater than that at pH 3 or 3.5, where most of the 5 mM anion is in the divalent form. Furthermore, judged from temperature at which turbidity appeared at pH 0.5, the effect continues down to that pH. This suggests that the monovalent bisulfate is equally as effective as divalent sulfate. Phosphate, however, which is monovalent throughout the range of the sulfate effect has little effect on the T_m at any pH (see Figure 1).

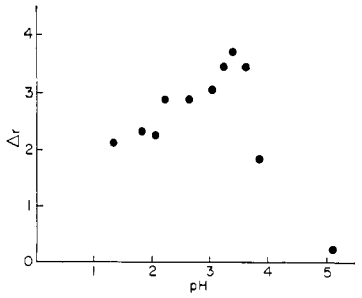

FIGURE 2: Calculated values of Δr, the number of protons bound to an unfolded molecule in excess of those bound to a folded one, as a function of pH.

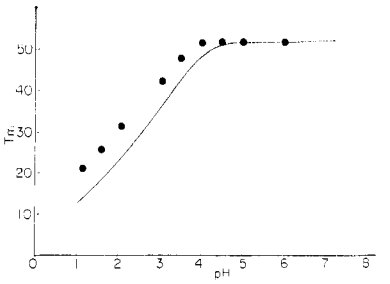

FIGURE 3: Increased stability afforded by sulfate in the acid range. The line is that of Figure 1, representing T_m as a function of pH in $\Gamma/2 = 0.1$ ammonium chloride. The points were obtained with the inclusion of 5 mM sulfate in the same solvent.

There is very little change in the absorption spectra of either the folded or unfolded forms at pH 0.7 from those at neutral pH. In particular, the height of the difference peak at 286 mμ, that is, the decrease in absorption at that wavelength as the temperature is raised through the transition range, is the same at pH 0.7 as at pH 7.

In the alkaline range above about pH 9, the situation is more complex, in part because of the ionization of tyrosines, which depends not only on the pH, but also on the state of folding of the protein and which strongly affects the absorption spectrum of the protein. Figure 4 shows a difference spectrum between what is presumably the native structure and the unfolded form at pH 12.2 (in 0.033 M Na_3PO_4). It was necessary to add the sample to the solvent at near 0° and avoid warming before taking the first spectrum, as the spectral change which takes place with warming at this pH is only in

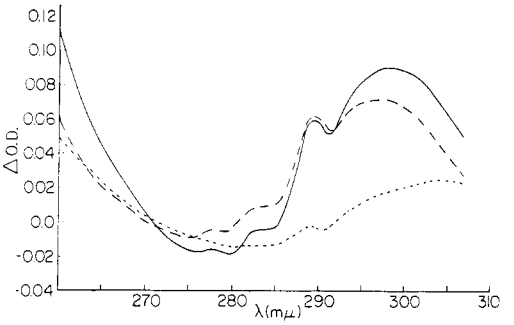

FIGURE 4: Difference spectra at pH 12.2. (——) Spectrum at 25° minus initial spectrum at 0°. (— —) Spectrum at 25° minus spectrum at 0° of recooled sample. (– – – – –) Spectrum of recooled sample minus initial spectrum. Both at 0°. All spectra were of one and the same sample in a sealed cuvet.

TABLE I: Transition Temperature of Barnase (0.2 mg/ml) in Various Solvents.

Solvent	T_m (°C)
pH 8.0, 0.1 M sodium bicarbonate	52.2
pH 8.0, 0.1 M sodium bicarbonate–2.0 M urea	38.8
pH 8.0, 0.1 M sodium bicarbonate–3.0 M urea	34.3
pH 8.0, 0.1 M sodium bicarbonate–4.0 M urea	24.4
pH 8.0, 0.1 M Tris-Cl–2.0 M guanidine hydrochloride	20.0
pH 8.0, 0.1 M Tris-Cl–6.0 M guanidine hydrochloride	<0
pH 9.0, 0.1 M sodium bicarbonate–3.0 M urea	33.5
pH 9.0, 0.1 M sodium bicarbonate–3.0 M urea–10^{-5} M Cu^{2+}	29.0
pH 9.0, 0.1 M sodium borate–3.0 M urea	33.4
pH 9.0, 0.1 M sodium borate–3.0 M urea–10^{-5} M Cu^{2+}	29.0
pH 9.0, 0.1 M Tris-Cl–3.0 M urea	33.5
pH 9.0, 0.1 M Tris-Cl–3.0 M urea–10^{-5} M Cu^{2+}	33.4
pH 8.0, 0.1 M Tris-Cl–10^{-5} M guanosine monophosphate	50.0[a]

[a] It was necessary to use only 0.1 mg/ml of barnase in this measurement because of turbidity which appeared at higher concentration as the T_m was approached.

part reversible. Also shown in Figure 4 are the differences between the initial spectrum and that of the recooled sample and that between the recooled sample and that taken at 25°. The last curve is equivalent to the difference between the other two. All three were derived from spectra run against air on one and the same sample in a sealed cuvet. The large extremum near 300 mμ is due, of course, to the ionization of tyrosines in addition to those ionized in the initial state. From the absolute values of the absorptions at 300 mμ, using data from model compounds given by Edelhoch (1967), I have calculated that only two tyrosines are ionized (at pH 12.2) in the initial state and all six in the unfolded form at 25°. The partial recovery on recooling may be accounted for by the deionization of one residue or by a change in the spectrum of the ionized chromophores if they were reburied without deionization. The smaller positive extremum at 291 mμ is due to exposure of tryptophans to a more aqueous environment, and it is clear that these groups are only partly buried in the recooled form.

When a sample of barnase which had been warmed to 25° at pH 12.2 was titrated back to pH 7.0 (with 1.0 N HCl) its ultraviolet absorption spectrum reverted to that characteristic of the native, folded form. Specific enzymatic activity was also that of the native enzyme.

Table I shows the T_m values obtained in a variety of other solvents. The interest in the effect of Cu^{2+} arose in some work on the effect of urea on elution volume of the enzyme on G-75 Sephadex gel filtration (Hartley, 1968). In preliminary experiments reagent grade urea was used without treatment. Later, in order to avoid the effects of cyanate contamination (Stark *et al.*, 1960), the urea was cleaned by passage through DEAE-cellulose (free base form). This treatment modified the effect of urea as shown in Figure 5. It was then found that the inclusion of 0.02 M potassium cyanate in the equilibrating solvent had no

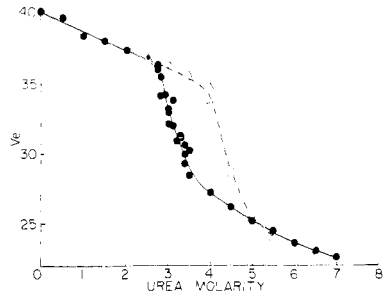

FIGURE 5: Chromatography of barnase on G-75 Sephadex in urea. V_e is the emergence volume; (●) points obtained using reagent grade urea, (△) points obtained using urea cleaned by passage through DEAE-cellulose (free-base form). The column was 183 × 0.63 cm; the solvent was 0.1 M sodium bicarbonate (pH 8.0) with the urea concentration shown on the abscissa. The temperature was 25°.

effect on the elution volume. Out of a survey of likely contaminants, including some 20 di- and trivalent cations, only the addition of Cu^{2+} was found to mimic the "dirty" urea. Cu^{2+} was effective at 10^{-6} M. Assay of the reagent grade urea for copper yielded 1.4 ppm, which would give about 5×10^{-6} M in 4 M urea solution. If NH_4HCO_3 or Tris-Cl were used in place of the $NaHCO_3$, copper had no effect, indicating that these copper complexing agents can compete successfully with the enzyme for the copper. Note, however, in Table I, 10^{-5} M Cu^{2+} does reduce the melting temperature in 3 M urea, 0.1 M $NaHCO_3$, although these solutions contained 1.5×10^{-3} M NH_4HCO_3, introduced with the sample. Copper was effective in lowering the V_e in 0.1 M sodium phosphate, but only above pH 7.

While the addition of 10^{-5} M guanosine monophosphate, an end product of RNA hydrolysis by this enzyme, had little effect on the T_m (Table I), it is interesting to note that the solubility of the unfolded form was reduced in its presence and it was necessary to use half the usual enzyme concentration in obtaining this value.

Discussion

In Figure 6 the pH dependence of the barnase transition is compared with that of bovine pancreatic ribonuclease. The similarity between these two curves argues strongly for similar mechanisms for the destabilization of the two enzymes by hydrogen ions and it seems clear, as Hermans and Scheraga

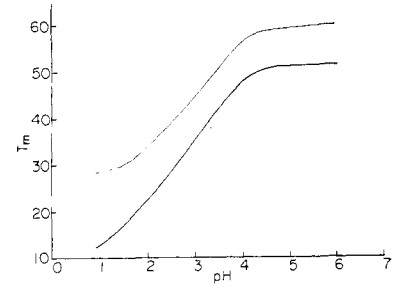

FIGURE 6: The pH dependence of the thermal transition of barnase (heavy line) compared with that of pancreatic ribonuclease (light line, taken from Hermans and Scheraga, 1961a).

(1961a,b) concluded for pancreatic ribonuclease, that the data cannot be completely accounted for by simple electrostatic effects accompanying discharge of normal carboxyl groups.

As the maximum difference (at about pH 3.5) in bound protons in Figure 2 is 3 or 4, the presence of a like number of abnormal carboxyl groups is indicated. While the reliability of the processed data of Figure 3 is not great, the sharp drop-off on the high pH side is consistent with normal pK's ($\sim$4.7) in the unfolded form (as might be expected), while the more gradual drop on the low end suggests that the abnormal pK's of the folded form are spread out in the pH 1–3 range. It is also possible that part of the higher value near pH 3.5 is due to the difference in nonspecific electrostatic interactions between folded and unfolded forms. While protonation of the abnormal groups favors the unfolded form, the leveling off of the T_m at very low pH, as with pancreatic ribonuclease, implies that Δr drops to zero and indicates that complete protonation is still compatible with native folding.

Hermans and Scheraga (1961b) have proposed that those carboxyl groups of pancreatic ribonuclease are involved in hydrogen bonds with the buried phenolic groups of tyrosines. The occurrence of four such buried tyrosines in barnase makes such an hypothesis attractive in this case also. It should be noted, however, that the spectral change which occurs on titration of pancreatic ribonuclease to low pH, while the temperature is kept below the T_m, does not occur with barnase. This change was attributed by the above authors to a change in the phenolic chromophore on the protonation of its associated carboxyl group. Another difference is the decline in ΔH with pH, which parallels the drop in T_m in barnase, but does not occur in the mammalian enzyme.

The effect of sulfate also has its precedent with pancreatic ribonuclease (Ginsburg and Carroll, 1965). The facts that stabilization occurs only below the pK of normal carboxyl groups and continues to a pH where almost all of the sulfate is in its singly ionized form suggest that the key reaction is hydrogen bonding to protonated normal carboxyl groups, rather than simply binding to positively charged groups. A sulfate or bisulfate ion may act to bind together two such carboxyl groups or one such and a positive group, thus stabilizing the native structure.

The complex behavior at high pH is difficult to interpret from the present data and further investigations are indicated. In particular it will be necessary to observe the transitions with parameters which do not depend so strongly upon the tyrosines alone.

The destabilizing effect of Cu^{2+} ion again has its parallel in pancreatic ribonuclease (Herzig and Bigelow, 1967). No effect on barnase was seen with Zn^{2+}, however, which also reduces the stability of pancreatic ribonuclease. By analogy with the pancreatic enzyme (Breslow and Girotti, 1966) and from the fact that the effect disappears below pH 7, binding of Cu^{2+} to one or both of the histidine residues is assumed.

In a previous paper (Hartley, 1968) I reported that the enzymatic activity of barnase in 3 M urea drops off sharply at a temperature ($\sim$34°) equal to the T_m as measured spectrophotometrically in the absence of substrate. Since it must be assumed that enzyme–substrate binding occurs, it was puzzling that substrate had not markedly stabilized the enzyme. The most reasonable explanation is that substrate binds equally well to the unfolded form. The behavior of the enzyme in the presence of 10^{-5} M guanosine monophosphate is interesting in relation to this question, since the reduced solubility of the unfolded form obviously reflects its interaction with the nucleotide. Direct measurements of the binding of this and other substrate analogs to native and unfolded barnase are needed. Its behavior is in sharp contrast to that of the pancreatic enzyme, which is strongly stabilized by phosphate and other substrate analogs, and by substrate itself (Sela *et al.*, 1957; Nelson *et al.*, 1962).

The analogies reported in this paper between barnase and pancreatic ribonuclease should not be interpreted as evidence of homology between the two enzymes. The amino acid compositions are quite different (Lees and Hartley, 1966) and preliminary studies (E. A. Barker and R. W. Hartley, work in progress) reveal no obvious similarities in sequence. In particular, the sequences about each of the two histidines are quite different from those about the two (His-12 and His-119; Crestfield *et al.*, 1963) involved in the active site of pancreatic ribonuclease. What is suggested, rather, is that the specific interactions involved (such as the presumed tyrosyl–carboxylate bonds) are of common occurrence in proteins. This is not surprising in view of the limited number of such types of interaction available and the evolutionary demand for stabilization of specific protein conformations.

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