Biochemistry

 Volume 18, Number 12 June 12, 1979

Steps in the Pathway of the Thermal Unfolding of Ribonuclease A. A Nonspecific Photochemical Surface-Labeling Study†

R. R. Matheson, Jr.,‡ and H. A. Scheraga*

ABSTRACT: The technique of nonspecific photochemical surface labeling is used to study the thermal unfolding of ribonuclease A at pH 5. The results indicate that the unfolding process consists of a minimum of three overlapping stages. Below 35 °C, there are localized changes which involve Met-13 as well as some Tyr and Ala residues. From other work on the ultraviolet absorption spectrum and proteolytic susceptibility of ribonuclease A, the latter residues are tentatively identified as Tyr-92 and Ala-19 and/or Ala-20. Between 35 and 40 °C, the outer shell of the ribonuclease structure which is most exposed to the solvent begins to unfold in a broad transition that is not complete until 70 °C. This stage overlaps the partial unfolding of a nonpolar core which is composed of Phe, Val, Leu, and Ile residues. This core unfolds between 60 and 70 °C. The unfolded form of ribonuclease A at 78 °C and pH 5 still has some residual structure. The observation that the nonpolar core of ribonuclease A becomes exposed to the solvent only incompletely and at a late stage of the unfolding process suggests that the nonpolar residues are important in the initial stage (nucleation) of protein folding. This is consistent with the concept that hydrophobic interactions dominate in the nucleation process [Matheson, R. R., & Scheraga, H. A., 1978), *Macromolecules 11*, 819]. The advantages and disadvantages of the nonspecific photochemical surface labeling technique as one for studying protein unfolding are examined.

The thermally induced reversible unfolding of RNase A[1] near pH 5 appears to be a cooperative transition between two states when examined by a number of techniques (for reviews, see Tanford, 1968, 1970; Richards & Wyckoff, 1971; Privalov, 1974). In particular, experiments which detect overall or averaged properties of the molecule (e.g., optical rotatory dispersion or enzymatic activity) reveal an apparently two-state process. A notable exception to this generalization is the observation that precise calorimetric and fast-reaction kinetic experiments are not consistent with a two-state process near pH 5 (Tsong et al., 1970; Privalov et al., 1973; Tiktopulo & Privalov, 1974; Baldwin, 1975; Hagerman & Baldwin, 1976), but that the intermediates are present at only very low concentrations. Burgess & Scheraga (1975) interpreted the results from a variety of investigations of the equilibrium properties in terms of a proposed pathway for the unfolding of RNase A. That pathway was subsequently found to be consistent with the results of experiments on the rate of release of C-terminal valine by carboxypeptidase A during the thermal transition (Burgess et al., 1975), of ESR measurements on the thermal transition of spin-labeled RNase A (Matheson et al., 1977a), and of a laser Raman spectroscopic study of the thermal transition at pH 5 (Chen & Lord, 1976).

In this paper, the technique of nonspecific photochemical surface labeling (Matheson et al., 1977b) is used to examine the thermal unfolding of RNase A at pH 5. The data are consistent with the proposed pathway of Burgess & Scheraga (1975) (only one slight modification of their scheme being suggested here) and with the calorimetric studies. Since this is the first protein-unfolding reaction to be studied by the nonspecific photochemical surface-labeling technique, the results will also be discussed in terms of their implications for the utility of the technique itself.

Experimental Section

The materials and methods used here have all been described previously (Matheson et al., 1977b), with the following additions. Solutions of RNase A were heated to 60 °C for 5 min immediately before use in order to dissociate aggregates (Crestfield et al., 1963). The nitrogen-saturated solutions of RNase A (1 μM) and NAP-taurine (250–300 μM) were adjusted to the desired ionic strength (in the range of 0.001 to 0.1 M) with KCl and a pH of 4.9–5.3 (measured at 25 °C) with dilute HCl. Samples (5.8 mL) were thermally equilibrated (at temperatures in the range of 12 to 78 °C) for 10 min and flash-photolyzed in the apparatus described previously (Matheson et al., 1977b). Three or four such samples, flashed

† From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853. *Received December 7, 1978.* This work was supported by research grants from the National Science Foundation (PCM75-08691) and from the National Institute of General Medical Sciences of the National Institutes of Health, U.S. Public Health Service (GM-14312).

‡ National Institutes of Health Predoctoral Trainee, 1974–1978.

[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; P_{1-13}, the 13-residue, N-terminal fragment of RNase A which is produced by the reaction of Met-13 with cyanogen bromide; T_m, the temperature at which a thermal transition is one-half complete.

0006-2960/79/0418-2437$01.00/0

at a given temperature, were pooled in order to obtain sufficient material for amino acid analysis.

In experiments designed to cleave the labeled polypeptide chain at specific sites, 2–5 mg of labeled RNase A was prepared as described above (by using 0.1 M KCl in all cases) and separated from reaction side products (products of inter-label reactions) by gel filtration on a 2.3 × 162 cm column of Sephadex G-25 (Pharmacia) eluted with 0.2 N acetic acid. Reaction of the resulting material with cyanogen bromide (Gross & Witkop, 1962) was carried out in 70% formic acid solution by stirring the labeled RNase A with a 30-fold molar excess of CNBr (Aldrich) at room temperature for 24 h. The reaction was stopped by lyophilization. Chromatography of the cleaved, labeled RNase A on the Sephadex G-25 column described above produced partial separation of fragment 1–13 (P_{1-13}) from the rest of the labeled RNase A in experiments where labeling was performed below 40 °C, and reapplication of the P_{1-13} fractions produced chromatographically homogeneous material. We estimate that no more than 20% of the theoretically possible yield of labeled P_{1-13} was ever obtained, and that the yield from RNase A which had been labeled at 40 °C or higher was undetectably small. Small yields were obtained, most probably because the bulky, negatively charged label (probably on Met 13) interferes with the cleavage reaction.

Attempts were made to digest labeled RNase A with trypsin at constant temperature in a pH stat (Radiometer TTT1). Labeled RNase A was dissolved in 0.01 M KCl, pH 7.9, at a concentration of 0.1–0.5 mg/mL and a temperature of 50–60 °C. Under these conditions, unlabeled RNase A is digested very efficiently by trypsin (Ooi et al., 1963). Addition of trypsin (Worthington) produced a negligible uptake of base (0.01 M KOH), indicating that no appreciable cleavage occurred over a period of 4 h, most likely because of the presence of the label near lysine and arginine sites. Thus, trypsin cleavage of RNase A which has been labeled extensively with NAP-taurine cannot be used to produce fragments that might have been useful for the identification of the labeled sites.

Results

It has been shown (Matheson et al., 1977b) that RNase A can be labeled to different extents (depending on the degree of unfolding) with the reactive aryl nitrene generated photolytically from NAP-taurine. Subsequent amino acid analysis can determine which residues are not attacked by the nitrene and, hence, monitor the extent of exposure of most residues in RNase A to the solvent. If a residue is labeled it must be accessible to the label (and solvent). However, if it is not labeled, no definite conclusions can be drawn because labeling is not 100% efficient, the extent of modification is not related in a simple way to either the type or degree of exposure of a residue, and only modifications which are not reversed by acid hydrolysis are detected (Matheson et al., 1977b).

The total numbers of all amino acids (except His and Cys) detected by amino acid analyses of the acid hydrolysates of samples of RNase A that had been labeled at various temperatures and ionic strengths are shown in Figure 1. His and Cys are not included because the reliability of their determinations was affected adversely by an artifact (unidentified) which overlapped the His peak and by losses of Cys from both hydrolysis (Rupley & Scheraga, 1963) and photolysis (Matheson et al., 1977b). A few labeling experiments were carried out at higher temperatures (up to 90 °C) and higher ionic strengths (up to 0.2 M KCl). However, these conditions proved unsuitable. At very high temperatures, the precision

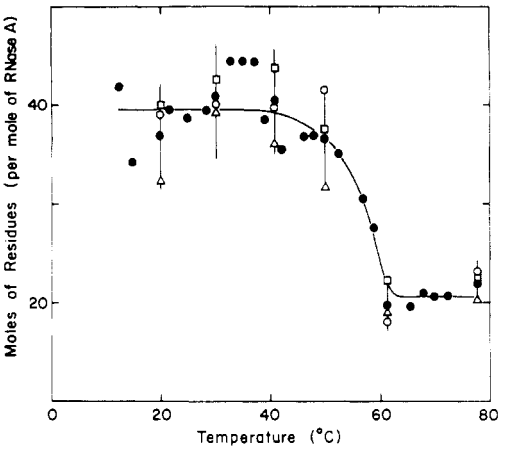

FIGURE 1: The total number of unmodified residues (moles/mole of RNase A) (excluding His and Cys) observed in the amino acid analyses of RNase A solutions which had been labeled at the indicated temperatures (±1 °C). The filled circles (●) represent the average number of residues observed at all ionic strengths. Individual totals for the analyses of material labeled at 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△) are shown for six representative temperatures. All labeling experiments were carried out on unbuffered solutions at pH 5.1 ± 0.2 (measured at 25 °C). The error symbols represent a standard deviation of ±13%.

of the amino acid analytical data was only about ±25%, probably because of leakages around the thermally expanded tygon end seals of the flashing apparatus. At higher ionic strengths, a desalting step was required before the sample could be hydrolyzed and subjected to amino acid analysis. This step contributed additional uncertainty to the quantitative determination of the amount of unmodified amino acids. However, within the larger error limits, no differences were evident between the number of residues labeled at 78 and 90 °C (0.01 M KCl) or between the number labeled at 0.1 M KCl and 0.2 M KCl (25 °C). The flatness of the curve in Figure 1 above 65 °C indicates that the main unfolding transition appears to be over by 78 °C. This does not mean that the protein is completely unfolded at temperatures above 78 °C, but only that any residual folded structures are not observed to unfold, by our technique. Increases in ionic strength above about 0.2 M at pH 6.5 alter the transition temperature of RNase A (Hermans & Scheraga, 1961). Thus, the range of conditions for the experiments summarized in Figure 1 is probably sufficient for a complete description of the transition at pH 5 and low ionic strength. The particular contributions of each type of amino acid to the sums plotted in Figure 1 are listed in Table I for two representative analyses (pH 5, 25 and 78 °C). For convenience in later discussion, Table I also includes some previously published analyses for RNase A labeled under different conditions.

The amounts of unmodified Thr and Phe residues found in the acid hydrolysates of labeled RNase A are displayed in Figure 2 as a function of the temperature and ionic strength conditions of labeling. Temperature profiles qualitatively similar to that shown for Thr (dependent on ionic strength and sensitive to temperature changes below the overall T_m of 56 °C) were observed for Asp, Ser, Glu, Pro, Ala, Met, Lys, and Arg. Temperature profiles qualitatively similar to that of Phe (independent of ionic strength and independent of temperature below about 56 °C) were observed for Val, Leu, and Ile. Gly and Tyr were somewhat exceptional in that, whereas their temperature profiles were independent of ionic strength, the number of unlabeled Gly residues was nearly independent of temperature over the entire temperature range. Also, the number of unlabeled Tyr residues was sensitive to temperature

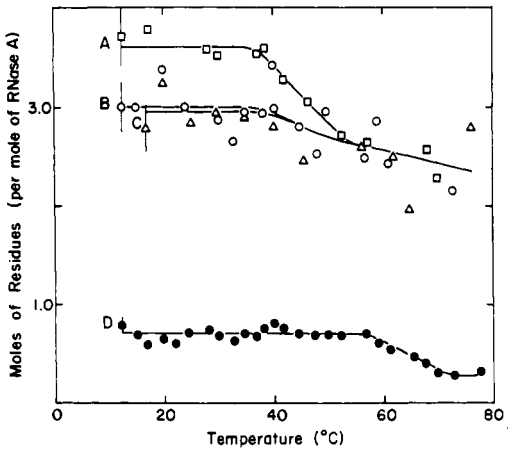

FIGURE 2: The number of unmodified residues (moles/mole of RNase A) of Thr or Phe observed in the amino acid analyses of RNase A solutions which had been labeled at the indicated temperatures (±0.3 °C). The numbers of Thr residues are shown as a function of temperature for 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△). The number of Phe residues are shown (●) for these same ionic strengths, but only an averaged value is given since the number of Phe residues is independent of ionic strength. The error symbols represent the observed standard deviation for three determinations of Thr (8.2%) and Phe (7.1%).

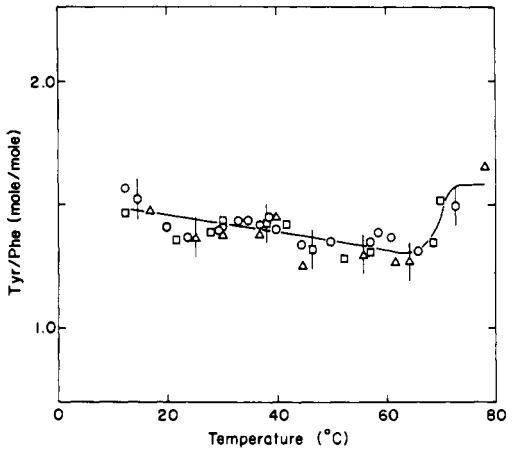

FIGURE 3: The ratio of the number of moles of unmodified Tyr to the number of moles of unmodified Phe observed in the amino acid analyses of RNase A which had been labeled at the indicated temperatures (±0.3 °C) in 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△). The error symbol represents an uncertainty of ±5.2% (standard deviation).

over the entire temperature range; i.e., the curve for Tyr did not show evidence of a sharp transition over a limited temperature range.

The insensitivity of Gly to changes in labeling conditions is probably a result of its small side chain which is labeled only with difficulty under all conditions. Thus, any change in solvent exposure would produce only minute changes in the extent of modification of Gly, and these cannot be distinguished from the experimental uncertainty. The behavior of Tyr is not so easy to explain, and will be considered in the Discussion section. In order to clarify the behavior of Tyr, as well as to characterize the temperature profiles of other residues more precisely, we have determined ratios of amounts unlabeled for particular pairs of amino acids. By computing relative areas of two peaks from the same chromatogram, the uncertainties arising from sample preparation and handling are eliminated. Figures 3–6 present four such ratios of the areas of the peaks for selected pairs of amino acids. Figure 3 compares Tyr and Phe. The curve in this figure shows that, although there are hints of discontinuity at 16 and 43 °C, a

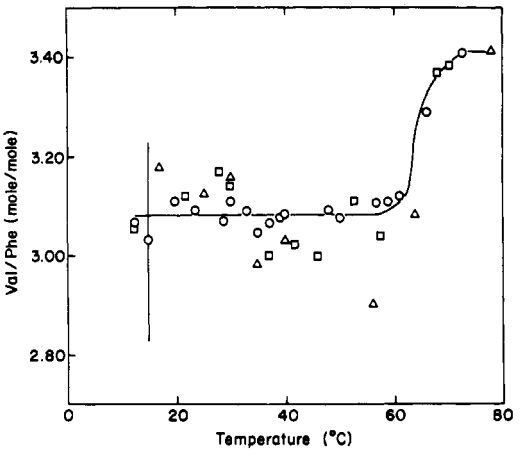

FIGURE 4: The ratio of the number of moles of unmodified Val to the number of moles of unmodified Phe observed in the amino acid analyses of RNase A which had been labeled at the indicated temperatures (±0.3 °C) in 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△). The error symbol represents an uncertainty of ±5.0% (standard deviation).

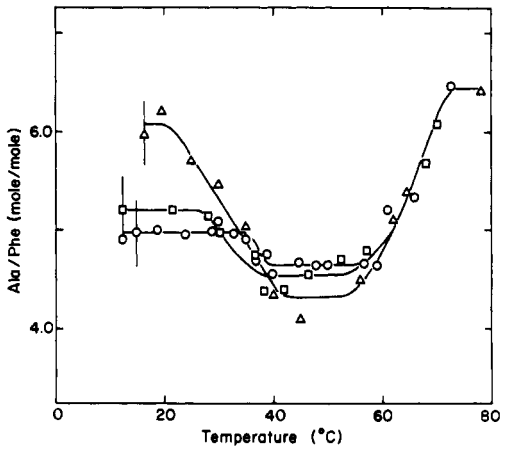

FIGURE 5: The ratio of the number of moles of unmodified Ala to the number of moles of unmodified Phe observed in the amino acid analyses of RNase A which had been labeled at the indicated temperatures (±0.3 °C) in 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△). The error symbol represents an uncertainty of ±6.0% (standard deviation).

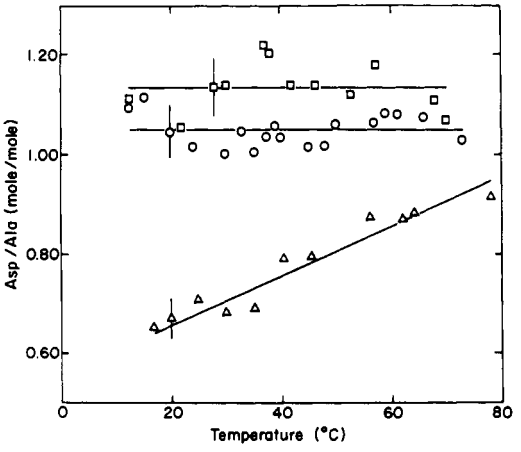

FIGURE 6: The ratio of the number of moles of unmodified Asp to the number of moles of unmodified Ala observed in the amino acid analyses of RNase A which had been labeled at the indicated temperatures (±0.3 °C) in 0.001 M KCl (□), 0.01 M KCl (○), and 0.1 M KCl (△). The error symbols represent an uncertainty of ±5.5% (standard deviation).

single straight line can describe the data from 12 to 60 °C adequately. Between 65 and 68 °C, the ratio of Tyr to Phe

Table I: Amino Acid Analysis of Ribonuclease A Samples Flashed with 0.1 M KCl and NAP-taurine (moles of amino acid/mol of protein)

	RNase A				reduced RNase A[c]
amino acid[a]	25 °C, pH 5	25 °C, pH 6[b]	78 °C, pH 5	70 °C pH 3[b]	25 °C, pH 3
Asp (15)	3.1	5.4	2.0	2.6	2.1
Thr (10)	2.8	3.0	2.8	3.1	3.0
Ser (15)	4.1	5.3	2.8	3.8	3.5
Glu (12)	5.1	6.3	3.1	5.5	2.5
Pro (4)	1.1	1.1	0.9	0.8	0.3
Gly (3)	2.8	2.1	2.2	2.0	1.3
Ala (12)	4.2	4.2	2.2	2.4	2.0
$^1/_2$-cystine[d] (8)	1.4	2.0	<0.2	0.7	0.4
Val (9)	2.4	2.6	1.0	1.3	1.2
Met (4)	0.8	1.0	0.4	0.4	0.4
Ile (3)	0.9	0.6	0.4	0.4	0.3
Leu (2)	1.4	0.7	0.5	0.4	0.4
Tyr (6)	1.0	1.1	0.4	0.5	0.4
Phe (3)	0.7	1.0	0.3	0.4	0.3
His[d] (4)	1.1	0.9	<0.2	0.7	0.4
Lys (10)	3.1	3.5	0.9	2.3	1.6
Arg (4)	0.4	0.2	0.4	0.2	0.4
Total[e]	34	38	20	26	20

[a] Numbers in parentheses are the numbers of residues in the native molecule (Richards & Wyckoff, 1971). [b] Data of Matheson et al. (1977b). [c] Data of Chavez & Scheraga (1977). [d] As discussed in the text (see Results), the data for Cys and His are subject to more uncertainty than those for the other residues. The observed values are included in this tabulation but should be regarded as only approximate. Values for Cys are reported as "half-cystine" even though the disulfide bonds were intact during hydrolysis. [e] Excluding the residues contributed by Cys and His (see Results).

increases. This is due to the larger decrease in area of the Phe peak between 65 and 70 °C (see curve D, Figure 2). Figure 3 can be compared with Figure 4 to illustrate how Tyr differs from Val. The ionic strength independent ratio of Val to Phe is a constant until between 62 and 65 °C where it undergoes a sharp increase, whereas that of Tyr varies continuously over the whole temperature range. While the area of the Val peak decreases in the temperature range of 62 to 65 °C, it does not decrease as fast as that of the Phe peak; hence, the ratio of the two increases. Ala exhibits a markedly different type of behavior. As mentioned above, the temperature profile of Ala is qualitatively similar to that of Thr. In Figure 5, the plots of the ratios of the areas of the Ala and Phe peaks clearly demonstrate the ionic strength dependence of the labeling of Ala (at low temperatures) and the onset of changes in this labeling at quite low temperatures (ca. 30 °C). The labeling of Met was also observed to change between 30 and 40 °C in a manner analogous to that of Ala. It is of interest to compare Figure 5 with the behavior of Thr described in Figure 1. Around room temperature, the extent of labeling of Ala decreases with increasing ionic strength whereas that of Thr increases. A plausible explanation for this is that, in the compact native structure of RNase A, local electrostatic interactions play an important role in orienting the negatively charged label and thus determining the extent of modification. Hence, the changes in intercharge screening induced by changes in ionic strength may affect the relative labeling of different residues in a variety of ways. Interestingly, the effect of ionic strength disappears at high temperatures (see Figures 1, 2, 5, and 6). Apparently, the thermally unfolded protein is sufficiently extended and structurally loose so that neither interlabel nor label–protein electrostatic interactions influence the labeling detectably.

Recently, Bayley & Knowles (1978) have pointed out that our experiments may produce modification of the protein by a mechanism other than the nitrene attack and the ultraviolet-damage mechanisms which we have considered. They suggest that the high concentration of diradical nitrenes, which are produced by the flash, will abstract hydrogen atoms from the water to a significant extent and, thus, give rise to a large number of hydroxyl free radicals which can attack the protein and result in intramolecular cross-linking and other processes. Since such secondary attack would also result in the modification of the amino acid residues, our method of analyzing by difference from unlabeled RNase A (rather than by isolating and identifying the actual reaction products) cannot distinguish between residues "labeled" by the nitrene and those "modified" by secondary free radical reactions. As a result, our apparent "efficiency of labeling" (i.e., the fraction of nitrene molecules which react directly with the protein) will be anomalously high. Because of the purpose to which our data are put, our conclusions will be rather insensitive to the presence of additional reactive species in the *external* solution. We are interested in using the reaction with nitrene as a gauge of how solvent exposure *changes* with unfolding. Thus, the absolute amounts of any residue which are labeled (by whatever free radical) are of only marginal interest, as compared with the changes in the extent of modification that accompany unfolding. Increasing the exposure of a residue to solvent must surely change its probability for reacting with reactive material(s) in the solvent whatever they are. Thus, by observing only the numbers and types of *unmodified* residues, we can draw conclusions about changing solvent exposure regardless of what reaction actually occurs to modify them.

All the results presented thus far pertain only to the total number of amino acids of various types found in the digests of labeled and totally hydrolyzed RNase. In order to test a proposed pathway of folding such as that of Burgess & Scheraga (1975), information about specific residues is required. Unfortunately, our attempts to produce and isolate specific fragments of labeled RNase A have been largely unsuccessful. CNBr has proved to be the only useful cleavage reagent, and only over a very limited temperature range and for only one fragment (P_{1-13}). The analyses of labeled P_{1-13} which were obtained from RNase A labeled below 40 °C are given in Table II. The amounts of each amino acid found are given relative to Thr, which is arbitrarily set equal to 0.20 in each case.

Examination of Table II reveals that the extent of modification of the results of P_{1-13} (relative to that of Thr) changes in a rather complex fashion (presumably because of a conformational change) between 20 and 40 °C. The extent of modification of Phe-8 is rather constant, but that of Glu, Ala, His, and Arg show minima, and the extent of modification of Lys goes through a maximum at 30 °C. Since the overall extent of modification of RNase A is constant over this temperature range (see Figure 1), whatever conformational fluctuations are responsible for these changes in the extent of modification of P_{1-13} must be localized. However, they involve residues near His-12 of the active site of RNase A. Thus, they may be important, as far as the mechanism of enzymatic action and its temperature dependence are concerned. The practical difficulties of small yields and rather poorly reproducible results for labeled P_{1-13} prevent any but very tentative conclusions about the nature of such a local conformational change. The results presented in Table II should not be taken as good evidence for a local conformational change in RNase

Table II: Amino Acid Analyses of the P_{1-13} Fragment of Labeled RNase A

amino acid[a]	20 °C	24 °C	30 °C	37 °C	40 °C
Thr (1)	0.20[b]	0.20[b]	0.20[b]	0.20[b]	0.20[b]
Glu (3)	2.0	2.0	3.0	0.88	0.16
Ala (3)	0.02	0.02	0.06	0.36	0.24
Hse (1)	c	c	c	c	c
Phe (1)	0.20	0.20	0.20	0.18	0.14
Lys (2)	1.4	1.2	0.12	0.36	0.66
His (1)[d]	0.16	0.20	0.50	0.06	0.08
Arg (1)	0.14	0.20	0.50	0.18	0.04
total (13)[b]	4.12	4.02	4.58	2.22	1.52

[a] The numbers in parentheses are the number of residues of each type in P_{1-13}. [b] The amount of Thr is arbitrarily set equal to 0.20 and the other residues are expressed relative to this reference residue. The uncertainty associated with these values is approximately ±30%. [c] The homoserine (lactone) peak overlapped that of ammonia. This prevented the determination of the amount of homoserine. [d] None of these chromatograms showed the extraneous peak that usually produced distortion of the His peak. Thus, we have reported data for His in this table, even though we are not able to exclude the possibility that a small amount of this extraneous material may be present.

A between 20 and 40 °C, and further evidence for such a conformational change is presented elsewhere (Matheson & Scheraga, 1979).

The negative results obtained in the attempted tryptic digestions of the modified RNase A are almost certainly due to the presence of the bulky, negatively charged label near the normal sites of tryptic cleavage. All of the Lys and Arg residues are rather accessible in the crystal structure of RNase S (Richards & Wyckoff, 1971) as are the residues immediately following them in the sequence (except for Gln-11 and Cys-40 which follow Arg-10 and Arg-39, respectively). Thus, extensive modification near the normal sites of tryptic cleavage is quite probable and accounts for the failure to observe tryptic digestion.

Discussion

The nonspecific photochemical surface-labeling technique used in this work shows an apparently two-state transition in the total number of residues which are unmodified when flashed with the aryl nitrene label (see Figure 1). The midpoint of this transition is 56 ± 3 °C, and the transition region extends from about 40 to 60 °C. These results are in good agreement with the data available from other studies (Hermans & Scheraga, 1961; Foss, 1961; Privalov et al., 1973; Chen & Lord, 1976), but the large experimental uncertainty in our data prevents a compelling case from being made for or against a two-state process.

If we consider the changes in surface exposure of *individual* amino acids, a markedly different picture of the unfolding process emerges. Figure 2 shows that the number of unreactive Thr residues in RNase A begins to decrease between 35 and 40 °C and then declines at a rate which depends on ionic strength until very high temperatures. The nine types of residues which behave in this fashion are Asp (Asn), Thr, Ser, Glu (Gln), Pro, Ala, Met, Lys, and Arg. These comprise 69% of the residues in RNase A. All of the hydrophilic residues fall into this group and the nonpolar residues (Pro, Ala, and Met), which are also included, are all exposed significantly to solvent in the RNase S crystal structure (Richards & Wyckoff, 1971). Only Met-30 appears to be totally inaccessible in the crystal structure, but earlier studies have demonstrated that Met-30 is still accessible to modification in the native molecule (Glick et al., 1967; Goren & Barnard, 1970a,b). The degree of labeling of Tyr begins to change at very low temperatures, but it does not show the dependence on ionic strength that is characteristic of the other nine types of residues whose degree of labeling begins to change early in the thermal transition. The degrees of exposure of the six Tyr residues differ considerably (Li et al., 1966), and labeling of Tyr residues would be expected to occur over a wide range of temperatures. Thus, all of the types of residues that have labeling patterns that are sensitive to ionic strength and begin to change below the overall T_m of 56 °C show significant solvent exposure. Conversely, all of the types of residues which are generally exposed to the solvent in the crystal structure begin to change their labeling patterns early (i.e., below 56 °C) in the unfolding transition (Gly is exceptional; see Results) and, except for Tyr, have temperature profiles which depend on ionic strength. We conclude that the changes in the structure of RNase A that take place below 56 °C reflect changes in the residues which are (on the average) already near the surface in the compact native structure. These residues may be denoted as "shell" residues, because they are found only rarely completely in the solvent-inaccessible core of the crystal structure of RNase S (Met-30, Ser-90, and Tyr-97 being the few "buried" ones) and account for nearly all of the residues in the solvent-accessible layer which surrounds the core.

Tyr, Met, and Ala are unique among the shell residues in that, at an ionic strength of 0.1 M, they show significant changes in modification below 35 °C. The environment of the *side chain* of Tyr-92 is known to change with very little perturbation (Bigelow & Sonenberg, 1962; Li et al., 1966). Thus, we may infer that Tyr-92 is responsible for the changing extent of tyrosine modification at low temperature.

The changes in the degree of modification of Met and Ala were not expected from any studies that had been carried out previously. Since no other residues appear to be involved in this early stage of the unfolding transition (except for the suggestion of changes in the exposure of the residues of P_{1-13}, which is presented in the Results), these changes must involve residues already quite accessible to the solvent. The steadily declining yield of P_{1-13} in the temperature range of 20–40 °C suggests that Met-13 may be responsible for most of the change in Met. Richards & Wyckoff (1971) observed that the side chain of Met-13 can be rotated about its C^{α}–C^{β} bond so that it extends out into the solvent without major disruption of other residues. Such a rotation provides a plausible explanation for an increase in the degree of modification and, hence, a decrease in the efficiency of CNBr cleavage.

The changes in the degree of modification of Ala can reasonably be attributed to conformational fluctuations involving Ala-19 and Ala-20. These residues are quite exposed as evidenced by the ability of subtilisin to cleave the Ala-20–Ser-21 peptide bond in the native molecule (Richards & Vithayathil, 1959). The sequence from residues 17 to 25 is the one postulated by Burgess & Scheraga to unfold in stage II (35–45 °C). The evidence that they cite in support of this conclusion indicates that the sequence is unfolded easily, but cannot rule out a change in the Ala residues below 35 °C. Moreover, one of the important stabilizing interactions on the N-terminal side of the sequence which unfolds during stage II is the nonpolar contact between Met-13 and Val-47, and our results suggest that this hydrophobic interaction may be weakened above 30 °C. Thus, we would suggest that stage II of the pathway proposed by Burgess & Scheraga be expanded both in temperature range and in amino acid sequence. Instead of extending from 35 to 45 °C and spanning residues

17–25, it should extend from 30 to 45 °C and include residues 13–25. Indeed, it is certainly consistent to place Asp-14 in the same stage of unfolding as Tyr-25 since the two are hydrogen bonded to one another (Li et al., 1966; Wyckoff et al., 1970). For consistency, then, stage V of the Burgess–Scheraga scheme, which formerly spanned residues 1–16, must be shortened to include only residues 1–12.

The minor modification just suggested for the Burgess–Scheraga pathway is the only one called for by our results. Changes in the rest of the shell residues are consistent with the sequential but overlapping unfolding of the stages outlined by Burgess & Scheraga, but our results do not provide a critical test of their proposal. Principally, this is because our results reflect only changes that are averaged over all the residues of a particular type. It is of interest to note that the calorimetric studies reveal a predenaturational increase in heat capacity below about 40 °C, which has been attributed by Tiktopulo & Privalov (1974) to "a loosening of the protein structure at its weak points". This is followed by the major increase in heat capacity which accompanies the unfolding and commences near 40 °C at pH 5. These observations correlate very well with our results. The "weak points" in the RNase A structure appear to include the environment of the side chain of Tyr-92 and the exposed section between residues 13 and 25. The main unfolding transition, i.e., the one which is associated with the large increase in heat capacity (Tiktopulo & Privalov, 1974), appears to involve an initial loosening of the structure formed by residues at or near the surface. Only above the overall T_m does the degree of labeling of the bulky nonpolar residues, which are found in the RNase core, begin to change.

The modification of Phe, Val, Leu, and Ile changes in a comparatively sharp transition which overlaps only the last half or third of the transition of the shell residues. These four types of residues may be designated as the "core" residues and, except for Met-30, Ser-90, Tyr-97, and the cystine residues, are the only types found to be totally shielded from solvent in the crystal structure of RNase S (Richards & Wyckoff, 1971). The apparent individual T_m's (i.e., the T_m's determined from the labeling of individual residues) of these core residues are all 65 °C within experimental error, compared with the lower value obtained as an "average" over all stages of unfolding. Thus, at the resolution available from our results, the melting of the core of RNase A occurs in a single, cooperative step. The temperature profiles for the core residues are independent of ionic strength.

The degree of modification of neither core residues, shell residues, nor their sum ever approaches 100%. Figure 1 shows that, at the high temperature limit, there are about 20 residues that are still unmodified. Figure 1 and Table I show that this resistance is not primarily a result of electrostatic charge distributions (there being no dependence on ionic strength) or of the presence of cross-links (there being little change in labeling when the disulfides are reduced). It seems that there must be some structure in the thermally unfolded form of RNase A in order to account for the inaccessibility of so many residues to the solvent. Such structure could arise from nonnative hydrophobic bonding among the residues of the unfolded molecule or from persistent local sections of native structure such as those identified by Burgess & Scheraga (1975) (see also Tanford, 1968, 1970).

The detailed proposal of Burgess & Scheraga (1975) divides the unfolding process of RNase A into six stages. In stage I (15–35 °C) the environment of the side chain of Tyr-92 changes. In stage II (35–45 °C), the polypeptide chain from residues 17 to 25 folds out from the rest of the protein. Stage III (40–50 °C) involves the unfolding of the native structure of the polypeptide chain from residues 27 to 34 and from residues 75 to 80. Stage IV (50–60 °C) includes the unfolding of residues 51 to 60 and the C-terminal loop of residues 104 to 124. Stage V (55–65 °C) consists of the unwinding of the N-terminal residues 1–16, and stage VI (60–70 °C) involves the unfolding (perhaps only partial unfolding) of the remaining structure which involves residues 35 to 50, 62 to 74, and 81 to 102. The results reported here suggest that stage II involves more residues than originally proposed by Burgess & Scheraga (1975) and covers a slightly larger temperature range. Our suggestion that the accessibility to solvent of the fragment P_{1-13} may also begin to change early in the thermal unfolding (below 40 °C) supports the observation of Chavez & Scheraga (1977) that the N-terminal portion of RNase A (which unfolds in stage V) becomes distorted between 40 and 50 °C as judged from antibody-binding experiments. Apparently, the solvent accessibility of residues which comprise fragment P_{1-13} can be altered (as judged either by nonspecific surface labeling or by antibody binding) well before the structure of the polypeptide backbone itself unfolds. This behavior is similar to that of Tyr-92 whose side-chain environment changes at a much lower temperature than does the polypeptide backbone in its vicinity (in stages I and VI, respectively). In summary, the modified form of the Burgess-Scheraga proposed pathway for the unfolding of RNase A, which is consistent with all available evidence, consists of the six stages: I (15–35 °C), Tyr-92 side chain unfolds; II (30–45 °C), residues 13–25 unfold and the exposure to solvent of residues 1–12 begins to change; III (40–50 °C), residues 27–34 and 75–80 unfold; IV (50–60 °C), the conformation of residues 51–60 and 104–124 is altered from that in the native enzyme; V (55–65 °C), residues 1–12 unfold; and VI (60–70 °C), residues 35–50, 62–74, and 81–102 unfold.

One of the principal reasons that the steps in the pathways of protein folding are of interest is that they may provide information about the interactions that determine how the protein folds. Since a random search of all accessible conformations will not permit an initially unfolded protein to fold to its correct native structure in a reasonable time (Levinthal, 1966; Wetlaufer & Ristow, 1973; Anfinsen & Scheraga, 1975), most interest has centered on the early steps of the folding process. Presumably, the interactions that are operative in these early steps must restrict the conformational freedom of the protein considerably. Burgess & Scheraga (1975) have reasoned that the stretches of polypeptide chain of RNase A that unfold only at very high temperatures (if at all) provide the structures which constitute the nucleation sites for RNase A folding. There are three such stretches (stage VI). Starting from the hypothesis that hydrophobic interactions dominate in the nucleation process, Matheson & Scheraga (1978) have proposed a method for identifying nucleation sites in proteins. This method is based on the assumption that a stretch of polypeptide chain which can fold into a pocket (*not necessarily the native conformation of the stretch*) that maximizes hydrophobic bonding (not necessarily the *total* free energy) *and also enhances folding of other parts of the polypeptide chain* can be identified as the nucleation site. They predicted that the stretch of polypeptide chain from residues 106 to 118 is the primary nucleation site for RNase A. Some discussion of these two proposals is of interest.

The circumstantial evidence for a definite pathway in protein folding is very strong (Burgess & Scheraga, 1975, and references therein; Creighton, 1977a). The nucleation site must

exist early in the folding pathway since, otherwise, it cannot accelerate much of the folding process. Moreover, as indicated above, it is obligatory that the nucleation site must restrict the conformational freedom of part of the remainder of the unfolded protein since, otherwise, the time required for complete folding would not be reduced significantly by nucleation. We may say that the intact nucleus (and similar nuclei in other segments of the chain) *directs* later folding. These two properties which characterize nucleation sites (viz., relatively great thermal stability, which may arise partially from hydrophobic bonding, and the capacity to direct later folding) are not equally accessible to experimental study. The thermal stability of the native conformation of a particular segment can often be measured conveniently. Because of the flexible nature of even native proteins (Cooper, 1976), we can expect that some differences might exist between the native conformation of the nucleation site and its preferred conformation *during* the initial folding steps; however, it is unlikely that these conformations will differ significantly once the folding process has proceeded to any appreciable extent (Burgess & Scheraga, 1975). Thus, the relative thermal stabilities of various segments of a polypeptide chain can be measured and used to identify *potential* nucleation sites, not *all* of which will necessarily direct further folding. Burgess & Scheraga (1975) made the simplest assumption that the regions of RNase A which showed the *greatest* thermal stability were, in fact, the nucleation sites. However, this simple assumption neglects the second criterion (Matheson & Scheraga, 1978) that the nucleation site(s) must direct later folding of other parts of the chain. It is not easy to measure unambiguously the capacity of a particular (presumably near-native) conformation of one segment of a polypeptide chain to influence the conformational freedom of another segment, and we now turn to an examination of this problem.

The apparent induction of an increased amount of native structure in one *fragment* of a protein by the presence of a second fragment has been demonstrated for several proteins, including RNase A (Kato & Anfinsen, 1969; Gutte et al., 1972; Lin et al., 1972). Additionally, studies have been published in which one (Friedman et al., 1966) or many (Gutte, 1975) residues in RNase A are modified or deleted and the folding of the resulting analogues has been examined. Two factors are chiefly responsible for the difficulties which arise in interpreting these and similar experiments. First, the amount of native structure that is observed frequently depends on the technique used to measure it and, second, it is generally impossible to tell if the fragments or modifications produce their effects by interactions during the nucleation step or during other stages of folding. Some progress can be made if it is *assumed* that a particular conformation of the nucleation site is necessary and must be preserved unaltered if significant folding is to be observed. This assumption permits the positive identification of those segments of the polypeptide chain that are *not* nuclei for folding; however, nuclei and other segments which are necessary for *later* folding steps (or those which markedly influence the parameter used to monitor folding) cannot be distinguished.

This line of reasoning now permits us to identify nucleation sites tentatively by an extension of the process used by Burgess & Scheraga (1975). The pathway for folding that they proposed for RNase A (and that, in principle, can be elucidated for any protein with enough experimental effort) identifies those segments of the polypeptide chain with relatively great thermal stability. For illustrative purposes, let us suppose that those segments which unfold at or above the overall T_m of RNase A (stages IV–VI) are stable enough to meet the *first* criterion of nucleation sites. Studies of fragments and analogues can now be examined for information which might permit the elimination of some of these stretches from consideration as nucleation sites. For example, Kato & Anfinsen (1969) and L. G. Chavez, Jr., and H. A. Scheraga (unpublished results) have observed that significant folding occurs in the fragment 21–124. Thus, we conclude that, even though the N-terminal α helix is quite stable (it unfolds in stage V), it cannot be a nucleation site. Presumably, RNase A does not use this terminal α helix to direct the folding of other, less thermally stable regions of the native structure.

At least one of the three regions (residues 62–74) of RNase A of greatest thermal stability (Burgess & Scheraga, 1975) is part of a sequence (58–73) which can be eliminated without complete loss of folding to an enzymatically active structure (Gutte, 1975). While some judgment is required in order to decide whether there is enough native structure in this analogue to justify its designation as "folded", it is at least doubtful that this stable region is required for folding of RNase A. This analysis limits the possible centers of nucleation for RNase folding to one or several of the sequences of residues 35–50, 81–102, and 104–124. It does not contradict the proposed pathway of Burgess & Scheraga, but it suggests that their identification of all (and only) the sections of greatest thermal stability as "nucleation sites" may require modification.

An independent and, as yet, unverified proposal for the location of the nucleation site(s) in RNase A is that of residues 106–118 (Matheson & Scheraga, 1978). This segment of polypeptide chain is placed in stage IV of the Burgess-Scheraga scheme. The thermal stability of the native conformation of this segment is, thus, not among the greatest, but it is reasonably high. [It should be pointed out that the evidence for the unfolding of residues 104–124 in stage IV (Burgess & Scheraga, 1975) comes from observations on the behavior of residues 104, 105, 119, and 121–124, which are *outside* of segment 106–118. No *direct* observations on the unfolding of residues 106–118 are available. Thus, the inference that they unfold at this point may be modified by later work.] The predominantly nonpolar character of this section (which is postulated to be its significant feature) is preserved in homologous sequences (Matheson & Scheraga, 1978), and in analogues (Gutte, 1975) and fragments (Burgess & Scheraga, 1975, and references therein) which fold. Thus, both criteria for a nucleation site are met by this segment of the polypeptide chain. More experimental evidence is required to substantiate or disprove this proposal.

Thus, the suggestions for the location(s) of the nucleation sites for the folding of RNase A, proposed by Burgess & Scheraga (1975) and by Matheson & Scheraga (1978), are different because they assign different properties to a nucleation site. Neither proposal is in conflict with available experimental evidence except for the identification of 62–74 as a nucleation site, which is not easily reconcilable with the ability of Gutte's (1975) 70-residue analogue to fold. The differences accurately reflect our present level of knowledge about the properties of nucleation sites and protein folding, and further work is required to resolve them.

The study of Gutte (1975) mentioned above is instructive because it demonstrates that some folding is possible even when all the disulfide cross-links are eliminated from a naturally cross-linked protein. Also, L. G. Chavez, Jr., and H. A. Scheraga (unpublished results) showed that reduced RNase has a conformation quite similar to that of the native protein. This is persuasive evidence that the formation of disulfide

cross-links is not necessary for folding (even though disulfide bonds stabilize the final, i.e., native, structure). The formation of disulfide bonds in reduced RNase A has been studied extensively (Hantgan et al., 1974; Takahashi & Ooi, 1976; Creighton, 1977b). All the investigations agree that there is very little initial preference for particular disulfide bonds to form, and Creighton (1977b) also observes that the formation of particular disulfide pairings is not accelerated later in the process. Takahashi & Ooi (1976) find some indication that the pairing of Cys-65 and Cys-72 is the (slightly) preferred first native pairing. They, therefore, suggest that formation of the Cys-65-Cys-72 loop is the nucleation step for folding. However, as they point out, their proposal is inconsistent with Gutte's observation that folding can occur even when this section of the chain is eliminated.

The results of the nonspecific photochemical labeling study reported here are consistent with the picture of nucleation proposed by Matheson & Scheraga (1978). While the results cannot prove that residues 106–118 are, in fact, the nucleation site for RNase A, they do indicate the dominance of nonpolar interactions in the latter stages of unfolding. As discussed by Burgess & Scheraga (1975), it is quite plausible that the latter stages of thermal unfolding correspond to the early stages of folding. Thus, since the nonpolar core of RNase A becomes accessible to the solvent late in the transition and is only incompletely exposed even at very high temperatures, it can reasonably be inferred that this indicates that the formation of hydrophobic contacts is important both in the unfolded protein and in the nucleated protein. These are exactly the criteria used by Matheson & Scheraga (1978) in the process which resulted in the prediction that residues 106–118 constitute the nucleation site for RNase A folding.

To summarize, the results of this nonspecific photochemical surface labeling technique are interpretable in terms of a simple physical model that is consistent with a slightly modified form of the Burgess & Scheraga (1975) pathway. Following a slight loosening (below 35 °C) of the structure of RNase A in the vicinity of a Tyr residue (probably Tyr-92 from other evidence) and in the sequence around Met-13 and some Ala residues (probably 19 and 20 from other evidence), the unfolding proceeds in two overlapping but distinct phases. Beginning at about 40 °C, the surface of RNase A begins to loosen up, and modification of residues on or near the surface increases. The breakdown of the structure in this outer shell of the protein continues in a broad transition which is complete at about 70 °C. Beginning at about 60 °C, the bulky, nonpolar residues which form the core of the RNase A structure begin to become accessible to the label. The destruction of the structure of this core is more cooperative than that involving the shell residues and involves the simultaneous partial unfolding of the core in a 10 °C temperature interval. The final, unfolded form probably has some local compact structure.

Implications

Several insights into the process of protein unfolding have resulted from this study. They have explicitly confirmed a number of previously published conclusions about the unfolding process, in particular, that the origin of the predenaturational changes in heat capacity lies in local fluctuations of mostly exposed residues (Tiktopulo & Privalov, 1974), and that residual structure exists in the thermally unfolded protein (Aune et al., 1967; Burgess & Scheraga, 1975). A new observation which results from this work is that the thermal unfolding is at first localized in the outer shell of residues and, only when this unfolding is substantially complete, does the inner, nonpolar core begin to break down. This conclusion appears to have been anticipated by Ikegami (1977) and Kanehisa & Ikegami (1977).

It is appropriate to examine the technique of nonspecific photochemical surface labeling and its usefulness for probing protein unfolding processes. Except in the most favorable situations (e.g., the Tyr-Phe comparison of Figure 3), changes in even the ratios which can be measured precisely are only slightly greater than the experimental uncertainty. For most amino acids, the precision is not high enough to detect intermediate states of folding. Moreover, there are difficulties in interpreting the observation that some residues remain unlabeled, and there may be problems from photolysis artifacts (Matheson et al., 1977b; Bayley & Knowles, 1978). The photolysis artifacts did not influence the analysis of the results for RNase A, because they were small and localized and they affected only a minor fraction of the residues in the total acid digests. For other proteins, where artifacts due to photolysis may be more extensive, or where they may affect a large fraction of the residues in a particular isolatable fragment, their presence may cause difficulties. Finally, the presence of the label in the polypeptide sequence creates problems with cleavage procedures. Against these disadvantages can be set the ability to detect changes in the structure involving residues such as Thr and Val. This advantage would appear to be marginal except in a protein where only a few such residues are present and where the residues are affected by the unfolding process to a considerable degree. The three Phe residues in RNase constitute such a favorable situation. However, this may not hold, in general, for the small proteins, which are usually the targets of unfolding studies.

Finally, it should be emphasized that none of the nonspecific photochemical surface-labeling results obtained in this study of RNase A would be easy to interpret or particularly convincing without the vast quantity of other data on RNase A which are available in the literature. Thus, the conclusion must be that the nonspecific photochemical surface labeling technique should not be the first choice for investigating protein folding. For an overall view of the process, calorimetry is more precise and does not suffer the severe limitations of the nonspecific photochemical surface labeling technique (viz., that no denaturants or buffers can be used which would react with the label, and extremes of pH must be avoided). However, without additional data, one cannot relate the calorimetric observations to processes occurring at specific sites in the molecule. For a detailed picture of an unfolding process, the laborious accumulation of many different experimental results is a necessity. Nonspecific photochemical surface labeling is one of the techniques that can contribute to this accumulation of information, especially in detecting differences in the temperature ranges in which different types of residues become exposed; in this sense, evidence is provided for the existence of intermediates in the unfolding/folding pathway even though such intermediates are not present at a high enough concentration to be detected by the currently available physicochemical techniques.

Added in Proof

A report by Howarth (1979) that appeared after this manuscript had been submitted for publication has provided partial confirmation of the main observations presented here. Howarth studied the thermal unfolding of RNase A with ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The primary points of accord are: (1) clearly at pH 3.1, and less so at pH 5.5, residues of Ala, Asn, Thr, Tyr, and Ser appear to unfold early in the thermal transition; (2) at pH values in the range of 3.1–6.6, Ile, Met Phe, and His unfold about 5–10

°C above the main transition temperature T_m; (3) the unfolding of the C-terminal region (which includes our predicted nucleation site, 106–118) exhibits a steeper temperature dependence than the main transition; (4) considerable local structure exists in the denatured state.

Another useful comparison can be made between the present work and that of Howarth. NMR spectroscopy, in principle, can distinguish between structures that fluctuate on the time scale of the NMR experiment and those that fluctuate either much faster or much slower. Such a distinction cannot be made by the nonspecific surface-labeling technique. Thus, our data cannot be used to distinguish stable nucleation sites unambiguously from regions of compact associations that are labile on the time scale of the lifetime of the nitrene.

Finally, for reasons stated in the first paragraph of the Results section, the nonspecific surface labeling technique cannot characterize the composition of the thermally stable regions, as the NMR method can. Elucidation of the specific location of the nucleation site(s) requires additional study, e.g., by the immunological method (Chavez and Scheraga, 1977) or by the ^{13}C NMR method (Howarth, 1979).

Acknowledgments

We are indebted to T. W. Thannhauser for carrying out the amino acid analysis and for determining the precision of ratios of areas of peaks for pairs of amino acids.

References

Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205.
Aune, K. C., Salahuddin, A., Zarlengo, M. H., & Tanford, C. (1967) *J. Biol. Chem. 242*, 4486.
Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453.
Bayley, H., & Knowles, J. R. (1978) *Biochemistry 17*, 2414.
Bigelow, C. C., & Sonenberg, M. (1962) *Biochemistry 1*, 197.
Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403.
Burgess, A. W., Weinstein, L. I., Gabel, D., & Scheraga, H. A. (1975) *Biochemistry 14*, 197.
Chavez, L. G., Jr., & Scheraga, H. A. (1977) *Biochemistry 16*, 1849.
Chen, M. C., & Lord, R. C. (1976) *Biochemistry 15*, 1889.
Cooper, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2740.
Creighton, T. E. (1977a) *J. Mol. Biol. 113*, 295.
Creighton, T. E. (1977b) *J. Mol. Biol. 113*, 329.
Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 618.
Foss, J. G. (1961) *Biochim. Biophys. Acta 47*, 569.
Friedman, M. E., Scheraga, H. A., & Goldberger, R. F. (1966) *Biochemistry 5*, 3770.
Glick, D. M., Goren, H. J., & Barnard, E. A. (1967) *Biochem. J. 102*, 7C.
Goren, H. J., & Barnard, E. A. (1970a) *Biochemistry 9*, 959.
Goren, H. J., & Barnard, E. A. (1970b) *Biochemistry 9*, 974.
Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856.
Gutte, B. (1975) *J. Biol. Chem. 250*, 889.
Gutte, B., Lin, M. C., Caldi, D. G., & Merrifield, R. B. (1972) *J. Biol. Chem. 247*, 4763.
Hagerman, P. J., & Baldwin, R. L. (1976) *Biochemistry 15*, 1462.
Hantgan, R. R., Hammes, G. G., & Scheraga, H. A. (1974) *Biochemistry 13*, 3421.
Hermans, J., Jr., & Scheraga, H. A. (1961) *J. Am. Chem. Soc. 83*, 3283.
Howarth, O. W. (1979) *Biochim. Biophys. Acta 576*, 163.
Ikegami, A. (1977) *Biophys. Chem. 6*, 117.
Kanehisa, M. I., & Ikegami, A. (1977) *Biophys. Chem. 6*, 131.
Kato, I., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 1004.
Levinthal, C. (1966) *Sci. Am. 214* (No. 6), 42.
Li, L. K., Riehm, J. P., & Scheraga, H. A. (1966) *Biochemistry 5*, 2043.
Lin, M. C., Gutte, B., Caldi, D. G., Moore, S., & Merrifield, R. B. (1972) *J. Biol. Chem. 247*, 4768.
Matheson, R. R., Jr., & Scheraga, H. A. (1978) *Macromolecules 11*, 819.
Matheson, R. R., Jr., & Scheraga, H. A. (1979) *Biochemistry 18* (following paper in this issue).
Matheson, R. R., Jr., Dugas, H., & Scheraga, H. A. (1977a) *Biochem. Biophys. Res. Commun. 74*, 869.
Matheson, R. R., Jr., Van Wart, H. E., Burgess, A. W., Weinstein, L. I., & Scheraga, H. A. (1977b) *Biochemistry 16*, 396.
Ooi, T., Rupley, J. A., & Scheraga, H. A. (1963) *Biochemistry 2*, 432.
Privalov, P. L. (1974) *FEBS Lett. 40*, S140.
Privalov, P. L., Tiktopulo, E. I., & Khechinashvili, N. N. (1973) *Int. J. Pept. Protein Res. 5*, 229.
Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647.
Rupley, J. A., & Scheraga, H. A. (1963) *Biochemistry 2*, 421.
Takahashi, S., & Ooi, T. (1976) *Bull. Inst. Chem. Res., Kyoto Univ. 54*, 141.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1.
Tiktopulo, E. I., & Privalov, P. L. (1974) *Biophys. Chem. 1*, 349.
Tsong, T. Y., Hearn, R. P., Wrathall, D. P., & Sturtevant, J. M. (1970) *Biochemistry 9*, 2666.
Wetlaufer, D. B., & Ristow, S. (1973) *Annu. Rev. Biochem. 42*, 135.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305.