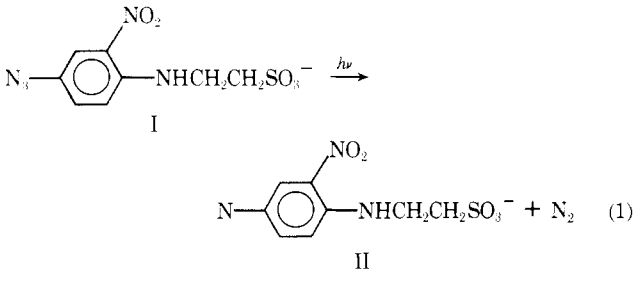
Study of Protein Topography with Flash Photolytically Generated Nonspecific Surface-Labeling Reagents: Surface Labeling of Ribonuclease A†

R. R. Matheson, Jr.,‡ H. E. Van Wart, A. W. Burgess, L. I. Weinstein, and H. A. Scheraga*

ABSTRACT: A method for *nonspecifically* labeling essentially all exposed residues of a protein is described. A reactive aryl nitrene is generated from *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-Taurine), within 500 μs by flash photolysis in the presence of protein. The reactive nitrene is inserted in about 2 ms into those carbon–hydrogen bonds of the protein that are exposed to the solvent. The method is applied here to ribonuclease A to demonstrate the different degree of labeling of the native and denatured protein. On the basis of amino acid analysis, it appears that residues of the native protein that are buried in the interior of the molecule (as judged from the x-ray structure) do not react with the nitrene. However, when these residues (even nonreactive ones such as valine and proline) are exposed by denaturation of the protein, they do react with the nitrene. It is shown that native ribonuclease A retains 90% of its enzymatic activity when flashed in the absence of NAP-Taurine. This small loss in activity arises from the disruption of a limited portion of the native enzyme structure, as judged by circular dichroism, ultraviolet, and Raman spectra. The site of this limited disruption may be a portion of the enzyme surface near the Cys-26-Cys-84 disulfide bond. The utility of this surface labeling technique for studying the pathways of protein folding or unfolding is discussed.

In order to carry out studies of the pathway(s) of protein folding an experimental technique is needed that can monitor and discriminate among changes in many regions of the protein structure. Techniques which monitor the degree of surface exposure of various residues fall into this category. Nearly all of these techniques depend on the observation of the relative exposure of *specific* residues or types of residues. However, Staros and Richards (1974) have demonstrated the significant role that *nonspecific* labeling can play in monitoring surface features of cell membranes. They investigated the degree of exposure of protein components of human erythrocyte membranes in the cell surface, thus extending the applicability of aryl nitrenes from photoaffinity labels (Fleet et al., 1972; Knowles, 1972) to general covalent labels. Staros and Richards (1974) accomplished nonspecific labeling (over a period of 20 min) by slow photolytic generation of the aryl nitrene *N*-(4-nitreno-2-nitrophenyl)-2-aminoethylsulfonate (II)



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which rapidly attacks exposed protein molecules, principally by means of insertion into carbon–hydrogen bonds.

Nuclear magnetic resonance (NMR)[1] spectroscopy (McDonald and Phillips, 1967; Meadows et al., 1968; Bradbury and King, 1972; Zaborsky and Milliman, 1972; Roberts and Benz, 1973), circular dichroism (CD) (Pflumm and Beychok, 1969; Simons et al., 1969) and optical rotatory dispersion (ORD) (Glazer and Simmons, 1965), ultraviolet (Shugar, 1952; Hermans and Scheraga, 1961; Brandts and Hunt, 1967), infrared (Hermans and Scheraga, 1960), and Raman spectroscopy (Lord and Yu, 1970; Chen and Lord, 1976), proteolytic digestion (Rupley and Scheraga, 1963; Klee, 1967), chemical modification of specific residues (Cha and Scheraga, 1963; Crestfield et al., 1963), and other techniques have been used to investigate the native structure and pathway(s) of folding of ribonuclease *in solution.* No one of the techniques is adequate for the description of the native structure. The results of many different experiments on the denaturation of a single protein can be considered together to obtain some insight on protein folding, but only with considerable experimental and deductive effort. The most detailed example of this type of analysis is the proposal by Burgess and Scheraga (1975) of a pathway for the thermal unfolding of bovine pancreatic ribonuclease A. To the extent that changes in the surface exposure of residues reflect conformational changes in the protein, the rapid, nonspecific labeling method introduced here provides additional useful information for such an analysis. It offers the advantages of the applicability to residues with relatively inert side chains (e.g., valine, proline, etc.) and the ability to probe nearly all of the surface of a protein instead of some limited area.

In this paper, a method is introduced which uses the nonspecific labeling properties of II to monitor the surface exposure of nearly all the amino acid residues of a protein molecule.

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‡ National Institutes of Health Predoctoral Trainee.

[1] Abbreviations used: NMR, nuclear magnetic resonance; CD, circular dichroism; ORD, optical rotatory dispersion; NAP-Taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; UV, ultraviolet; IR, infrared; TLC, thin-layer chromatography.

Flash photolytic generation of II in the presence of protein permits extremely rapid labeling of these exposed residues, which can be identified by subsequent amino acid analysis. Residues that are not labeled by the nitrene *may* be buried. This method is applied here to native and denatured bovine pancreatic ribonuclease A and holds promise for applicability to the study of protein unfolding.

Experimental Section

Materials. *N*-(4-Azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-Taurine) (I) was synthesized by the procedure of Staros (1974). The 90-MHz proton NMR spectrum (2.3% in deuterated Me_2SO) contains peaks at δ 7.75 (d, 1, phenyl position 3), 7.42 (q, 1, phenyl position 5), 7.15 (d, 1, phenyl position 6). The rest of the spectrum is in close agreement with that of Staros and Richards (1974). Our product is identical with the major component of commercial NAP-Taurine (Pierce Chemical Co.) as indicated by chromatography on thin-layer silica gel plates (E. Merck) in chloroform–methanol (1:1) and isopropanol–formic acid–water (20:1:5). The NAP-Taurine was stored as moist (with water) crystals at 4 °C in the dark. Although aqueous solutions of NAP-Taurine appeared stable (as judged by thin-layer chromatography) for about 2 days in stoppered volumetric glassware at room temperature and exposed to room light, care was taken to photolyze solutions containing this compound within 2 h of their dilution from freshly prepared, light-protected stock solutions.

Bovine pancreatic ribonuclease (RNase) was purchased from Sigma (Type II-A) and purified by column chromatography on carboxymethylcellulose (Whatman CM52) by the procedure of Taborsky (1959). Fractions corresponding to Taborsky's fraction D (RNase A) were pooled, dialyzed, lyophilized, and stored at 4 °C until needed. Enzymatic activity was determined with cyclic cytidine 2′,3′-phosphate (P-L Biochemicals) (see the Ribonuclease Activity section).

Denatured RNase A was obtained by heating dilute solutions of RNase A (pH 3, 0.1 M KCl) to 70 °C for 15–20 min. This was done in the thermally jacketed photolysis cell described in the Flash Photolysis Apparatus section.

Distilled deionized water was used throughout.

Ultraviolet–Visible, Circular Dichroism, and Raman Spectroscopy. All UV–vis spectra were obtained with a Cary Model 14 spectrophotometer. CD spectra were recorded with a CD 6001-equipped Cary Model 60 spectropolarimeter. The Raman spectra were obtained with an instrument described previously (Van Wart et al., 1976). The power at the sample was about 300 mW. An instrumental resolution of 7 cm^{-1} was used and, in a typical spectrum, data were collected for between 45 and 90 s at 2-cm^{-1} intervals.

Flash Photolysis Apparatus. Figure 1 shows a schematic diagram of the sample cell and flash lamp circuit. The radiation source is an I.L.C. 7L6 Xenon flash tube, which was fired at 2 kV and discharged in about 500 μs. The sample compartment is a cylindrical annulus of 1-mm path length, 15.2-cm long. A thermally jacketed cell (not shown in Figure 1) of comparable dimensions was used for work at temperatures other than room temperature. In a typical experiment, dry nitrogen gas (N_2) was blown through the compartment and bubbled through the sample for 15 min. After allowing 10 min for temperature equilibration, the sample was flashed, the volume of gas evolved was determined and the sample was recovered.

The production of nitrene was followed quantitatively by measurement of the volume of N_2 evolved, using a Warburg manometer (Dixon, 1951), since 1 mol of N_2 and 1 mol of reactive aryl nitrene are the anticipated initial products of photolyzed NAP-Taurine.

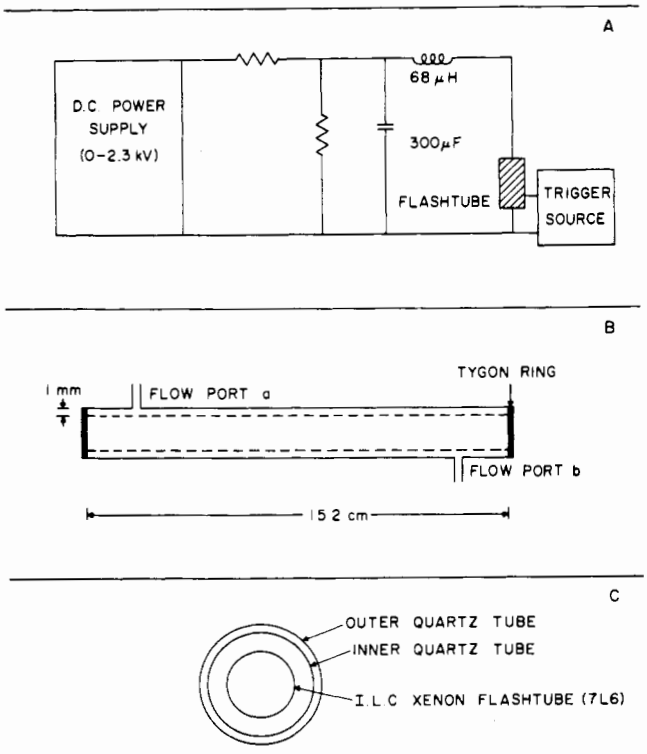

FIGURE 1: Diagram of the flash photolysis apparatus. (A) Schematic of the electronics; (B) dimensions of the sample cell; (C) end view of the sample cell and flash tube.

A uranyl oxalate actinometer was used to estimate[2] the dosage of light (moles of photons per unit volume of sample) provided in one flash.

Amino Acid Analysis of Labeled Protein. Nitrenes generated in the presence of protein are capable of insertion into carbon–hydrogen bonds to form secondary amines which are stable to acid hydrolysis (Reiser et al., 1966; Smith, 1970). Those amino acid residues which are so attacked will then possess a covalently bound label and an additional negative charge ($R\text{-}SO_3^-$, $pK \approx 2.5$). Hydrolyzates (6 N HCl, 105 °C for 24 h in vacuo) of the labeled protein will then show an apparent loss of certain residues when chromatographed on a TSM automatic amino acid analyzer. Following recovery of flashed ribonuclease samples (with or without label), D,L-norleucine was added as an internal standard.

The standard deviation for the determination of a single type of residue was about 2% for native, unflashed RNase A and about 6% for the labeled RNase A samples. Correction factors for hydrolysis losses were those of Rupley and Scheraga

[2] The xenon emission spectrum is continuous for wavelengths greater than about 225 nm and extends into the near IR. The spectrum is only weakly dependent on operating parameters of the lamp (Carlson and Clark, 1965); hence, a typical xenon spectrum was employed to estimate the fraction of light intensity emitted in any particular spectral region. Taylor (1971) has tabulated the quantum efficiencies for the uranyl oxalate actinometer at a number of discrete wavelengths in the range 208–438 nm. By assuming that these quantum efficiencies were valid in a small wavelength region around that at which they were measured and that radiation of a wavelength greater than 500 nm induced a negligible amount of oxalate oxidation, it was possible to roughly estimate the dosage of radiation in a particular wavelength range.

(1963).[3] The value of 12.0 mol/mol of protein was assigned to the average of the areas of the glutamic acid and alanine peaks in hydrolyzates of unflashed RNase A samples (Rupley and Scheraga, 1963).

Ribonuclease Activity. The enzymatic activity of RNase A toward cyclic cytidine 2′,3′-phosphate was determined by the procedure of Crook et al. (1960) when no label was present, and by the method of Josefsson and Lagerstedt (1963) when activity in the presence of NAP-Taurine was of interest. For this latter procedure, use was made of TLC silica gel plates prepared without fluorescence indicator (E. Merck).

Immunological Procedure. Pancreatic ribonuclease A (Sigma Type II-A) was used without further purification for the immunization of New Zealand White rabbits. Antiribonuclease antiserum was produced as described by Brown (1962).

Ouchterlony (1948) double immunodiffusion was performed on glass microscope slides overlaid with 2 ml of 0.7% Ionagar (Colab, Chicago Heights, Ill.) in 0.05 M barbital buffer (pH 8.6). Patterns were cut with a no. 2 cork borer and were allowed to develop for 24 h at room temperature. Plates were soaked overnight in 0.15 M NaCl and then stained with a 1% solution of Buffalo Black in 0.1 M acetic acid prior to photographing.

Miscellaneous Methods. RNase A concentrations were determined spectrophotometrically, using λ_{max} 278 nm and ϵ_{278} 9700 (Sage and Singer, 1962). NAP-Taurine concentrations were determined spectrophotometrically using λ_{max} 471 and ϵ_{max} 4730 (Staros and Richards, 1974).

pH measurements were made with a Radiometer Type PHM 4c instrument equipped with a Radiometer GK2302B combined electrode.

Irradiation of NAP-Taurine solutions was also performed for long times (of the order of minutes) with a G.E. AH6 mercury arc lamp (using interference filters to isolate light of wavelengths between 200 and 400 nm), with a G.E. Quartzline BRH lamp, and with the 488-nm line of a Coherent Radiation Model 52 argon ion laser (using a beam expander).

Results and Discussion

Requirements That the Surface Labeling Technique Must Meet. In order to be applicable to the study of protein folding, any nonspecific surface labeling technique must meet the requirements specified below:

(1) The labeling process itself should not alter the surface topography of the protein. This requirement would be satisfied if the reaction took place on a time scale that is fast compared with that required for the protein to unfold sufficiently to allow labeling of residues which are buried in the interior.

(2) The presence of compound I (eq 1) in solution before labeling should not alter the surface features of the protein.

(3) It should be demonstrated that residues known to be buried in the native protein are not labeled but can be labeled in the denatured protein.

(4) In order to correctly interpret the labeling patterns (i.e., the number of each type of residue that is labeled), it should be determined to what extent the labeling reagent is nonspecific.

In the following sections, the above requirements are discussed with reference to the labeling of bovine pancreatic ribonuclease A with NAP-taurine, using flash photolysis to activate the label.

Photolysis of NAP-Taurine. One of the attractive features of aryl azide labels is that they can be photolyzed with light of long enough wavelengths so that radiation damage to their targets can be minimized (Smith et al., 1962; Knowles, 1972; Fleet et al., 1972; Staros and Richards, 1974). However, in the cases where this feature has been used to advantage, the irradiation times (with light of wavelengths greater than about 400 nm) have been on the order of minutes (Staros and Richards, 1974) or even hours (Fleet et al., 1972). For the case of proteins in aqueous solution, it seems very unlikely that a useful number of covalent labels (especially when they bear ionic charges to increase their solubility in water) could be attached over a period of many minutes without inducing some change in the protein surface, even without any radiation effects. Thus, we sought to determine by how much the irradiation time for NAP-Taurine could be decreased.

We attempted to induce photolysis (as measured by nitrogen evolution), using the broad visible absorption band centered near 470 nm, by irradiation with the 488-nm line of an argon laser. Irradiation for times as long as 10 min at a power of approximately 1 W resulted in thermal heating but an insignificant amount of photolysis. Hence, the visible absorption band is not useful for accomplishing rapid photolysis. The stronger absorption with λ_{max} 270 (ϵ 2.2×10^4 M^{-1} cm^{-1}) lies in the range (250–310 nm) cited by Smith (1970) as that for the longest wavelengths of effective azide photolysis. That absorption under this band results in efficient photolysis was confirmed by irradiation of NAP-Taurine samples for short times (<30 s) with light of wavelengths between 200 and 400 nm.

On the basis of these results, the possibility of effecting photolysis with flash lamps emitting in the near UV was investigated. Xenon lamps produce a significant amount of light with wavelengths of 225–400 nm, although the bulk of their emission is at longer wavelengths. With our apparatus complete photolysis of the azide could be achieved only with concentrations less than about 0.5 mM; hence, all of our labeling experiments were carried out with NAP-Taurine concentrations of 0.2–0.3 mM.

Reactions available to II include insertion into protein (when present) or other molecules of azide or nitrene, reaction of nitrene with an unphotolyzed azido group, radical recombination to form an azo compound, and hydrogen abstraction from the solvent followed by radical recombination (Reiser et al., 1968). The final product mixture from such "side" reactions is probably complex. The recombination of azide radicals proceeds at a diffusion-controlled rate in organic liquids (Reiser et al., 1968). This reaction limits the mean lifetime of nitrenes to the range of 1–5 μs for our range of concentrations. It is reasonable to assume that the radical–radical combination is also diffusion controlled in 0.1 M aqueous salt, but, even if it is three orders of magnitude slower, the entire labeling process (flash plus nitrene reactions) occurs in less than 2 ms. To avoid the side reaction of nitrenes with unreacted azide molecules, and also to compensate for the absorption of light by the protein, the NAP-Taurine concentration was kept well below the maximum that apparently allows complete photolysis.

Effect of Flashing on Native Ribonuclease in the Absence

[3] In this initial paper, no effort was made to determine quantitatively how much Cys was converted to cystine, cysteic acid, etc., by means of the flash. Thus, our reported values for half-cystine are probably too low. This problem was not investigated here because the products of photolyzed cystine residues in RNase are still somewhat uncertain, especially for a polychromatic light source such as ours (Augenstein and Riley, 1964; Rathinasamy and Augenstein, 1968; Shafferman and Stein, 1974). Investigation of the products of tyrosine-mediated cystine photolysis, under the conditions used here, is now in progress.

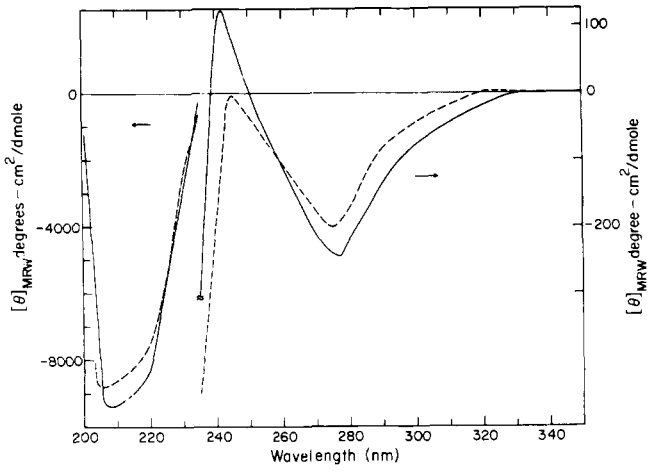

FIGURE 2: The circular dichroism spectra of native RNase A in 0.1 M KCl, 25.0 ± 0.2 °C, pH 5.96 (solid curve); flashed RNase A, same conditions (broken curve). Ellipticities are on a mean residue weight basis.

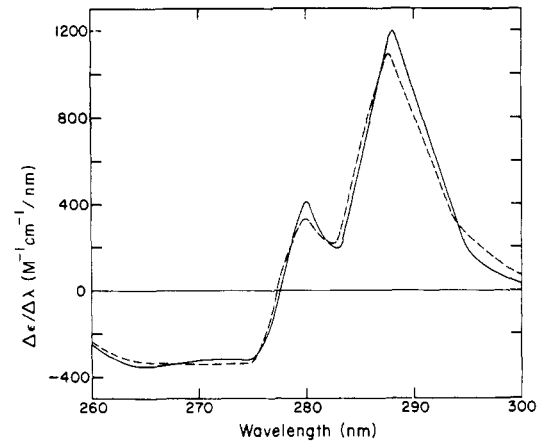

FIGURE 3: Ultraviolet difference spectrum of RNase A in 0.1 M KCl, pH 6.20 (solid curve), and flashed RNase A under similar conditions (broken curve).

of Label. It is well known that exposure of RNase A to UV light causes loss of enzymatic activity, conformational changes, and destruction of certain residues (Augenstein and Riley, 1964; Risi et al., 1967; Rathinasamy and Augenstein, 1968; Arian et al., 1970; Aktipis and Iammartino, 1971; Volkert and Grossweiner, 1973; Schultz et al., 1975; Laustriat and Hasselmann, 1975). In order to determine whether the flashing procedure used here caused such damage, samples were examined before and after flashing, using several different techniques.

RNase A samples exposed to the flash maintained 90% (±5%) of their original activity toward cyclic cytidine 2′,3′-phosphate.[4]

RNase samples, before and after flashing, were indistinguishable from one another with respect to their interaction with antiribonuclease antiserum. The double-diffusion experiments demonstrate that flashing under our conditions does not detectably disrupt the RNase A structure in the regions of the antigenic determinants.

The CD spectrum of RNase A before and after irradiation is shown in Figure 2. The spectrum of native RNase A is in substantial agreement with that of Pflumm and Beychok (1969) over the entire range of 200–340 nm. The CD of flashed RNase A differs from that of the native enzyme primarily above 235 nm. The negative band at 275 nm in native RNase A is attributed to contributions from tyrosyl residues and disulfide bonds (Pflumm and Beychok, 1969; Horwitz et al., 1970). The positive band at 242 nm has been ascribed by Simons et al. (1969) to "buried" tyrosines. Thus, the decrease in intensity of both of these bands upon irradiation can be interpreted as arising from disruption of disulfides, destruction of tyrosine residues, exposure of "buried" tyrosines, or some combination of these effects. This same interpretation has been offered by Aktipis and Iammartino (1971) to explain their data which are in qualitative but not quantitative agreement with ours since we retain about 90% of enzymatic activity after flashing. This difference in activity may be due to the difference in irradiation times in the two sets of experiments. In the region below 235 nm, native and flashed RNase A do not differ very much. Our results are similar to those of Schultz et al. (1975) for RNase retaining 70% or greater activity. We will not attempt to interpret the CD changes quantitatively in terms of loss of ordered structure, except to point out that our data provide no evidence for extensive structural changes due to irradiation.

The UV first-derivative spectrum of RNase A before and after flashing is shown in Figure 3. That of the native enzyme is in excellent agreement with that of Brandts and Kaplan (1973) (however, we did not correct for the very small disulfide contributions, as they did). The small blue shift of the crossover point (0.3 nm) implies that the contribution of buried tyrosines has been decreased relative to that of the exposed tyrosines. The shift is comparable to that (0.2 nm) seen in comparing the derivative UV spectra of RNase A with that of RNase S (Brandts and Kaplan, 1973). Flashed RNase A also shows the band broadening associated with spectral heterogeneity.[5] The most plausible explanation of these UV derivative spectra is that *one* of the "buried" tyrosines [Tyr-25, -92, or -97 (Woody et al., 1966)] has been altered chemically in some molecules (anticipating the amino acid analyses results) and/or "exposed" to the solvent by local denaturation.

Portions of the Raman spectra of RNase A before and after flashing are shown in Figure 4. The regions shown are those which provide the most direct evidence of the changes that may have occurred in the enzyme. Examination of the spectra between 400 and 1800 cm^{-1} shows no other major qualitative differences. The spectrum of unflashed, native RNase A is nearly identical with that of Yu et al. (1972) and Chen and

[4] It is of interest to compare this value with those of other workers who have studied the quantum yields for inactivation of RNase. Using the estimation procedure outlined in the Methods section, the dosage used here is about 3×10^{-7} einsteins/ml for λ between 225 and 250 nm, and 26×10^{-7} einsteins/ml for 250–300 nm. Rathinasamy and Augenstein (1968) have determined the following quantum yields for loss of enzymatic activity: 0.010 (229 nm); 0.030 (254 nm), 0.019 (265 nm), 0.007 (280 nm). Using an "averaged" value of 0.02 for all wavelengths in the range of 225–300 nm, and assuming that all light below 250 nm is absorbed by the sample and that light of 250–300-nm wavelengths is absorbed with an extinction coefficient of 8000, we obtain an exceedingly rough estimate of 12% inactivation, which compares well with the 10% mentioned above.

[5] Using the quantity $R\Delta\lambda_{1/2}$ [R = ratio of heights of the two peaks (at 290 and 280 nm), and $\Delta\lambda_{1/2}$ = half-width of the major peak], introduced by Brandts and Kaplan to characterize this broadening quantitatively, the flashed RNase A shows a value of 55.6 which is 42% of the way between the value of $R\Delta\lambda_{1/2}$ for native RNase (29.3) and the value of Brandts and Kaplan for thermally denatured RNase A (91.2). The magnitude of this increase in $R\Delta\lambda_{1/2}$ supports the interpretation that, if more than one of the tyrosine chromophores has been affected by irradiation, then the changes must be very small at each tyrosine.

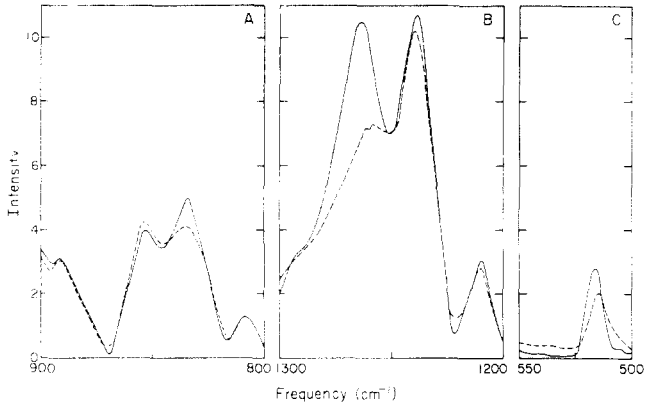

FIGURE 4: (A) Raman spectrum of RNase A in the region 800–900 cm^{-1} showing the tyrosine doublet, before (solid curve) and after (broken curve) flashing. The curves have been redrawn to eliminate noise and baseline differences (peak intensities in any spectrum are always relative to the intensity of the δ_{CH_2} vibration which is taken to be 10). (B) Raman spectrum of RNase A in the region 1200–1300 cm^{-1}, showing the amide III region before (solid curve) and after (broken curve) photolysis. (C) Raman spectrum of RNase A in the region 500–550 cm^{-1} before (solid curve) and after (broken curve) photolysis, showing the S–S stretch vibration. In this figure, all peak assignments were taken from Chen and Lord (1976).

Lord (1976). Figure 4A shows the tyrosine doublet which reflects the state of phenolic hydrogen bonding (Siamwiza et al., 1975). The differences between the samples before and after flashing imply the destruction or disruption of tyrosine residue(s). These differences are consistent with the exposure of buried tyrosine residues (Siamwiza et al., 1975), and the magnitude of the differences can be accounted for by changes in only one such tyrosine (Chen and Lord, 1976). As judged by the position and intensity of the peak of ca. 1239 cm^{-1} in the amide III region (Figure 4B), essentially no β structure is disrupted. The significant change in the peak of ca. 1260 cm^{-1} can be partly accounted for by changes in tyrosine residues (Frushour and Koenig, 1975), although other causes cannot be ruled out on the basis of the Raman results alone. The conformations responsible for intensity in this region have not yet been identified satisfactorily (Van Wart and Scheraga, 1977). Finally, Figure 4C shows a change in the S–S bond stretching region which demonstrates that the flash affects at least one of the S–S bonds of RNase A.

The amino acid analysis of RNase A samples before and after flashing (in the absence of compound I) are presented in columns 1 and 2 of Table I. Flashing causes no loss of residues other than half-cystine (2.1) and Tyr (0.7). These changes are in good agreement with the expectation based on previous work with UV-inactivated RNase A (Rathinasamy and Augenstein, 1968; Aktipis and Iammartino, 1971; Schultz et al., 1975). The disruption of the disulfides of RNase A by low dosages of radiation is nonrandom, with relative reactivities of 10:3:3:1 (Grist et al., 1965); thus, it seems very reasonable to assume that the two half-cystine residues destroyed in our flash are the two halves of the same cystine. There is a considerable body of evidence in the literature, which supports the conclusion that aromatic residues close to disulfides greatly enhance the photodisruption of these bonds (Augenstein and Riley, 1964; Rathinasamy and Augenstein, 1968; Shafferman and Stein, 1974). Two tyrosines (25 and 73) are adjacent to cystines (26–84 and 65–72) in RNase A (Richards and Wyckoff, 1971).

It can be seen from the evidence of activity measurements, immunological tests, CD, UV derivative, and Raman spectra, and amino acid analyses of flashed and unflashed RNase A that the flashing procedure does not have a major (irreversible) effect on the conformation of native RNase. Even if there were such an effect, the labeling experiment would still be unaffected since it is complete within 2 ms. Although some reactions of proteins (e.g., proton exchange or isomerizations) are known to occur faster than this, and others are potentially faster (in principle, conformational changes could occur on a molecular vibration time scale of about 10^{-12} s), the cooperative, multiresidue conformational changes associated with protein folding are probably slower. RNase A folding has been studied in detail by Baldwin and his colleagues (Tsong and Baldwin, 1972; Tsong et al., 1972; Garel et al., 1976), and the fast-folding step(s) that they have found in a number of relaxation experiments are characterized by relaxation times in the range of 10–100 ms near room temperature. The labeling reaction employed here is fast enough to avoid interference from folding steps of this speed. Thus, assuming that any gross unfolding due to the flash would also occur on this time scale, the labeling process would be complete before flash-induced effects could alter the labeling pattern, and requirement 1 can be met.

The effect of the flash on those specific light absorbing residues which are directly affected is probably much more rapid than the labeling reaction (Volkert and Grossweiner, 1973). It is important to identify such sites of primary photochemical processes so that any *localized* changes are not erroneously attributed to thermal unfolding during studies of the pathway(s) of protein unfolding. The CD, UV-derivative, and Raman spectra all indicate that a buried tyrosine residue is affected by the flash. The magnitudes of the spectral changes and the amino acid analyses suggest that only one such tyrosine is involved. The CD and Raman spectra also indicate the disruption of some cystine, which is limited to one of the four cystines by the amino acid analysis. Finally, the previously cited work of other investigators on the connection between aromatic side chains and radiation-disrupted disulfides focuses attention on structures in which the cystine is next to an aromatic residue in the primary sequence. Only one *buried* tyrosine (Tyr-25) is next to a Cys (Cys-26–Cys-84). All of this is consistent with the identification of Cys-26–Cys-84 as the initial disulfide disrupted by UV light and Tyr-25 as the chromophore chiefly responsible for its reactivity. Thus, we know the region in which to expect some uncertainty in identifying the unfolding behavior. In any case, the validity of the localization proposed here in no way affects the utility of the general surface labeling approach.

Effect of Unflashed Label in Solution with RNase A. Evidence that the presence of compound I does not denature the enzyme before flashing (requirement 2) comes from UV spectroscopy and activity measurements. The UV–vis spectrum of an unflashed solution of 0.06 mM NAP-Taurine and 0.05 mM RNase A in 0.1 M KCl at room temperature was exactly superimposable (within ±2%) on one generated by summing their respective spectra. Enzymatic activity in the presence of 0.5 mM NAP-Taurine was identical (within ± 10%) with that with no label present.

Amino Acid Analyses of Labeled RNase A. Much of the potential utility of nonspecific surface labeling for protein unfolding studies depends on its ability to provide information about the surface exposure of as many amino acid residues as possible. We will refer to the state in which every amino acid residue that is exposed to the solvent is labeled as "surface saturation." It must be shown (requirement 3) that residues, known from other experiments to be completely in the interior of the native protein, are not attacked when the protein is in

TABLE I: Amino Acid Analyses of Ribonuclease A Samples Flashed with NAP-Taurine (moles of amino acid/mole of protein).

Amino Acid[a]	1[b]	2[c]	3[d]	4[d]	5[d]	6[d]	7[d]	8[d]	9[d]	10[d]	11[d]	12[e]
Asp (15)	14.4	14.6	14.2	13.2	12.4	9.1	8.4	7.1	5.4	5.3	5.5	2.6
Thr (10)	9.9	9.9	9.4	9.6	7.7	5.6	5.0	4.3	3.0	2.9	3.0	3.1
Ser (15)	15.2	15.2	15.2	15.4	13.0	10.4	9.5	7.7	5.3	5.5	5.6	3.8
Glu (12)	12.3[f]	12.3	12.4	12.4	12.1	9.0	7.1	7.0	6.3	6.2	5.8	5.5
Pro (4)	3.9	3.9	3.6	3.6	2.9	2.4	1.9	2.0	1.1	1.1	1.0	0.8
Gly (3)	3.5	3.4	3.4	3.5	2.9	2.3	2.8	3.4	2.1	2.8	[g]	2.0
Ala (12)	11.7[f]	11.7	11.3	11.5	10.4	7.5	6.8	5.7	4.2	4.0	4.0	2.4
Half-cystine (8)	8.1	6.0	5.2	5.6	5.5	2.8	2.4	2.4	2.0	[g]	2.0	0.7
Val (9)	8.2	8.0	7.9	8.2	6.5	4.9	3.5	3.2	2.6	2.1	2.1	1.3
Met (4)	3.7	3.6	3.4	3.0	2.5	2.1	1.0	1.8	1.0	[g]	1.1	0.4
Ile (3)	2.1	1.9	1.9	2.0	1.3	1.0	0.9	0.9	0.6	0.6	0.6	0.4
Leu (2)	2.0	1.9	1.5	2.2	2.0	1.3	1.0	1.2	0.7	0.7	0.7	0.4
Tyr (6)	6.2	5.5	4.5	4.8	3.0	2.0	1.8	1.7	1.1	1.0	0.9	0.5
Phe (3)	2.9	2.9	2.9	2.5	2.6	1.6	1.3	1.6	1.0	0.8	0.6	0.4
Lys (10)	10.6	11.9	11.6	10.4	7.4	5.8	6.1	5.5	3.5	2.6	3.7	2.3
Arg (4)	3.2	3.6	3.5	3.1	2.0	1.4	1.4	1.2	0.0	0.2	0.2	0.2
His (4)	3.5	3.6	3.7	3.2	2.2	1.1	1.6	1.7	0.9	0.9	0.6	0.7

[a] Numbers in parentheses are the numbers of residues in the native molecule (Richards and Wyckoff, 1971). [b] Native ribonuclease. [c] Flashed ribonuclease in 0.1 M KCl, 25.0 ± 0.3 °C, concentration 630 to 0.7 μM. Same conditions as for all labeling experiments,[d] except that compound I was absent. [d] The concentration of NAP-Taurine was kept constant at about 0.29 mM. To achieve the label/protein ratios indicated, the concentration of enzyme was adjusted. Columns 3-11 pertain to labeled ribonuclease, with initial label to protein ratios of 4.8/1, 10/1, 20/1, 51/1, 106/1, 159/1, 212/1, 265/1, and 424/1 mol/mol, respectively. [e] Ribonuclease labeled at 70 °C in 0.1 M KCl, pH 3, using an initial label to protein ratio of 212/1. [f] Based on the assignment of 12.0 residues/mol of protein to the average of the Glu and Ala peaks. All other values are referred to this assignment as well (see Methods). [g] Less than two usable replicates available.

the native state, but are attacked when the protein is denatured (requirement 3). Additionally, the specificity of the label must be known in order to extract the maximum amount of information from the results obtained at surface saturation, or to help in understanding why surface saturation is apparently unattainable (requirement 4). Partial but very suggestive evidence of how close surface saturation can be approached is summarized in the amino acid analyses of Table I. For all residues except Gly, the amount of unlabeled amino acid decreases and then levels off with increasing label/protein ratio. The values at high label/protein ratios (>200/1) represent the maximum number of unlabeled residues in ribonuclease.

According to the data of Richards and Wyckoff (1971), there are 3 Val, 1 Tyr, 1 Phe, 1 Met, 1 Ser, and 4 half-cystine residues that are *completely* "buried" (both side chain and α-carbon) by the criteria of Lee and Richards (1971). If requirement 3 is to be met, *at least* these numbers and types of residues must be observed in all hydrolyzates. At high label/protein ratios, 2.1 Val,[6] 0.9 Tyr, 0.6 Phe, 1.1 Met, and 2.0 half-cystine are unlabeled. Column 2 of Table I shows that 2.1 half-cystine residues are modified by the flash itself, and subsequent information about them has been lost; thus, if the cystine modified by the flash was initially buried, only 2 half-cystine residues would be found near surface saturation. The physical measurements discussed in preceding sections (Effect of Flashing on Native Ribonuclease in the Absence of Label and Effect of Unflashed Label in Solution with RNase A) show that little denaturation takes place before labeling, so that it is reasonable to make the assumption that the disrupted cystine is an initially buried one. Thus, the first part of requirement 3 is fulfilled.

It remains to be shown that the residues presumed to be protected from the label in the native structure can be labeled in the denatured enzyme. Column 12 of Table I presents the results obtained from hydrolysis and amino acid analysis of RNase labeled at 70 °C, pH 3.0. Except for the case of Ser, all of the types of buried residues (Val, Tyr, Phe, Met, and half-cystine) were present in smaller amounts after flashing at 70 °C. Hence, the second part of requirement 3 is fulfilled.

Turning to requirement 4, columns 9, 10, and 11 of Table I show that surface saturation has not been attained. In addition to those 11 residues needed to satisfy requirement 3, there are 5 Asp (or Asn), 3 Thr, 5 Ser, 6 Glu (or Gln), 1 Pro, 2-3 Gly, 4 Ala, 4 Lys, and fractional amounts of Ile, Leu, Arg, and His remaining in the hydrolyzates. In order to satisfy requirement 4 and obtain the information necessary to explain these results, we attempted to determine the selectivity of the aryl nitrene quantitatively. Such compounds are known to be somewhat selective in their insertion into C-H bonds (Knowles, 1972). Flashing of solutions containing excess NAP-Taurine together with mixtures of the amino acids found as residues in RNase A resulted in some fraction of all residues being attacked. Basic amino acids were labeled to a somewhat greater extent than acidic amino acids; for the nonpolar amino acids, the degree of labeling increased with the size of the side chain. However, these results cannot be generalized to the case of protein labeling. For example, although all 3 glycine residues in native RNase A are exposed to the solvent, and it is possible to label about 50% of the glycine molecules in an equimolar mixture of free amino acids, only a fractional amount of the Gly residues in RNase A are labeled. The selectivity of the nitrene toward residues of a given type may be significantly

[6] Hydrolysis for longer times (72 h) than those routinely used resulted in an increase of the amount of valine (by 0.8 residue) and isoleucine (by 0.7 residue) recovered (accompanied by decreases in the amounts of the other amino acids recovered, due to increased hydrolysis losses) from hydrolyzates of both native RNase A and from protein labeled with a label/protein ratio (chosen as 212:1 in this experiment). Peptide bonds involving valine are often difficult to hydrolyze because of steric hindrance which is particularly severe in the case of the Ile-107-Val-108 bond. In fact, Val-108 is one of the three "buried" valines and Ile-107 is substantially shielded from solvent in the x-ray structure (Richards and Wyckoff, 1971). Thus, the increases in the amounts of these two types of residues with more severe hydrolysis conditions satisfactorily explain the apparently low valine recovery figures in Table I. There are 2.9 unlabeled valine residues as surface saturation is approached.

influenced by the protein environment around these residues. For example, electrostatic interactions with nearby charged residues may contribute to orienting the nitrene and enhancing or decreasing the degree of labeling of a given residue out of proportion to either its exposure, or its intrinsic reactivity toward the nitrene.

In order to satisfy requirement 4 minimally, however, less than full information about the label specificity is required. Column 12 of Table I shows the analyses obtained from hydrolyzates of RNase A labeled at 70 °C, pH 3.0, conditions where it is substantially unfolded. These data demonstrate that all types of residues in RNase A *can* be labeled. Apparently Gly, Ser, Asp (probably as aspartic acid rather than asparagine residues), and Glu (probably glutamic acid rather than amide) are labeled only very inefficiently. Nevertheless, while we cannot quantitatively determine the selectivity of NAP-Taurine toward the various amino acids, we can state that, since no amino acid has zero reactivity, any changes in labeling patterns (e.g., changes induced by progressively unfolding the protein) can be interpreted as reflecting some modification of the protein structure around the labeled residues. The exact nature of such a modification may not be easy to elucidate until requirement 4 is satisfied more fully.

Conclusions

Nonspecific, surface labeling of proteins (when initiated by flash photolysis of an aryl azide precursor) has been shown to be a convenient means of covalently labeling those residues that are exposed to the solvent. The prospect of extending the nonspecific surface labeling technique to studies of unfolded structures in proteins now seems possible. Insofar as alterations in the surface reflect the stages of protein unfolding, the technique can contribute information that will complement the information available by other methods. The acquisition of more extensive information by introducing proteolytic digestion (to locate precisely which residues are on the surface and hence labeled), temperature variation (to obtain "intermediate states of folding"), and other modifications are clear possibilities.

For the particular case of RNase A labeled with NAP-Taurine, the labeling reaction proceeds without difficulty and yields evidence that suggests that Cys-26–Cys-84 is the disulfide most easily disrupted by UV light.

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Isolation, Characterization, and Activation of the Magnesium-Dependent Endodeoxyribonuclease from *Bacillus subtilis*†

William F. Burke, Jr.,* and John Spizizen

ABSTRACT: A major endodeoxyribonuclease was isolated from a mutant of the transformable *Bacillus subtilis* 168. The magnesium-dependent endonuclease was purified approximately 750-fold to electrophoretic homogeneity. The enzyme had a molecular weight of about 31 000, as determined by gel filtration and polyacrylamide gel electrophoresis. The protein appears to be composed of two subunits. The nuclease was dependent on magnesium or manganese ions for hydrolytic activity. The purified nuclease degraded DNA from several species of *Bacillus,* as well as *Escherichia coli* DNA, alkylated, depurinated, and thymine-dimer containing *B. subtilis* DNA, and hydroxymethyluracil-containing phage DNA. The enzyme also hydrolyzed single-stranded DNA, although native DNA was the preferred substrate. However, the nuclease was unable to degrade ribosomal RNA. The cleavage products of the DNA hydrolysis have 5′-phosphate and 3′-hydroxyl ends. The enzyme could be activated in crude extracts by heat treatment or treatment with guanidine hydrochloride. The nuclease activity was inhibited by phosphate and by high concentrations of NaCl. A possible function for this endonuclease in bacterial transformation is discussed.

Bacterial transformation is a process of intercellular transfer of genetic information in which DNA molecules bind to competent recipient cells, penetrate the surface layers of those cells, and recombine with the recipient genome. In the process of uptake and penetration, high-molecular-weight duplex-transforming DNA attaches to the surface of competent *Bacillus subtilis* cells in an endwise manner and enters in a linear progressive fashion (Davidoff-Abelson and Dubnau, 1973). Competent cells then convert transforming DNA of higher molecular weight to double-stranded fragments which possess lecular weights of about 9×10^6. These fragments are rated by endonucleolytic cleavage in the periplasmic space. These double-stranded fragments are then converted to single-stranded fragments, probably during transfer across the membrane.

The fragmentation of DNA in the initial phases of transformation thus suggests the involvement of at least several nucleases. A number of deoxyribonucleases have been demonstrated in *B. subtilis* (Chestukhin and Shemyakin, 1972; Ciarrocchi et al., 1976; Hayase et al., 1975; Kageyama, 1970; Kanamori et al., 1974a, 1974b). Two endonucleases have been suggested as candidates for the double-stranded fragment generating enzyme. One is the manganese-stimulated endonuclease described by Scher and Dubnau (1973, 1976), and the other is the magnesium-dependent heat-activated nuclease first demonstrated by McCarthy and Nester (1969).

In an effort to characterize the magnesium-dependent endonuclease, a purification technique was developed for the

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