Side-Chain Cotton Effects of Ribonuclease*

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ABSTRACT: Chemical modifications, solvent, and pH variation suggest that the side-chain Cotton effect of native ribonuclease observed at neutral pH is associated with one or more of the buried tyrosyl residues. At pH 11, the ionization of surface tyrosyl residue(s) generates a second Cotton effect not apparent when the residue(s) is protonated. Hence, at such alkaline pH, both these two Cotton effects are present but unresolved. Acetylation of ribonuclease with *N*-acetylimidazole in aqueous solution modifies three tyrosyl residues without significant effect upon either enzymatic activity or the optical rotatory dispersion of the protein. In contrast, acetylation in urea, followed by its removal, modifies all six tyrosyl residues and abolishes both the activity and the side-chain Cotton effect. The optical rotatory dispersion of this modified protein and the kinetics of deacylation by hydroxylamine suggest that acetylation of the buried tyrosyl residues prevents the refolding of the enzyme on removal of urea. Deacylation restores both activity and the rotatory dispersion pattern to resemble those of native ribonuclease. Solvent variation further demonstrates the interdependence of the environment of the buried tyrosyl residues and the occurrence of the side-chain Cotton effect. At alkaline pH, the apparent midpoint of the side-chain Cotton effect shifts to longer wavelengths. Optical rotatory dispersion and circular dichroism of the native protein at near-neutral pH compared with such determinations at alkaline pH indicate that the ionization of surface tyrosyl residue(s) generates a new Cotton effect, in addition to that associated with the buried tyrosyl residues. The consequences of chemical modification of buried tyrosyl residues are discussed in terms of their role in determining secondary and tertiary structure and maintainance of the conformation of ribonuclease.

Studies of a number of proteins by optical rotatory dispersion (Simmons and Blout, 1960; Ulmer *et al.*, 1961; Beychok, 1965; Cathou *et al.*, 1965; Coleman, 1965; Glazer and Simmons, 1965a,b; Myers and Edsall, 1965; Ulmer, 1965; Ulmer and Vallee, 1965; Urry, 1965) and amino acid polymers (Fasman *et al.*, 1964, 1965) have recently demonstrated the widespread occurrence of side-chain Cotton effects between 250 and 300 mμ. Apart from their intrinsic significance, the origin of such Cotton effects is important for the interpretation of the remainder of the rotatory dispersion spectrum. Further, since these Cotton effects are conformation dependent, they constitute a sensitive means of evaluating the role of aromatic amino acids in the formation of the tertiary structure of proteins. The possibility that tyrosyl residues of proteins might be optically active has provided a new approach for the investigation of the structural and functional roles of tyrosine in enzymes.

Ribonuclease has previously served importantly to elucidate the structural and functional role of tyrosyl residues. The evidence supports the view that three tyrosyl residues of ribonuclease are "free" and exposed to the solvent, while three are "buried"[1] within the interior of the folded molecule (Scheraga and Rupley, 1962). The three buried tyrosyl residues have been thought to function in maintenance of the tertiary structure of ribonuclease through hydrogen-bond formation with aspartyl carboxyl groups (Scheraga, 1957, 1960), or, alternatively, through hydrophobic interactions (Tanford *et al.*, 1955).

Since ribonuclease does not contain tryptophan and the conditions for the selective visualization of its free and buried tyrosyl residues have been established, studies of optically active aromatic absorption bands of this enzyme might assist in delineating the role of free and buried tyrosyl residues in generating side-chain Cotton effects. In this regard we have previously reported on the use of *N*-acetylimidazole both for the identification of free tyrosyl residues in proteins (Wacker *et al.*, 1964; Riordan *et al.*, 1965a) and for the study of their role in enzymatic function (Simpson *et al.*, 1963; Riordan *et al.*, 1965b). The data obtained in this manner were consistent with those obtained by other experimental means.

The present study reports the effects of acetylation with this agent on the optical rotatory dispersion and enzymatic activity of ribonuclease. The effect of various nonaqueous solvents and pH upon the optical rotatory dispersion has also been examined. Finally, the circu-

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[1] The terminology here employed has been discussed previously (Riordan *et al.*, 1965a)

lar dichroism of the protein at neutral and alkaline pH has been evaluated. The data suggest that the aromatic side-chain Cotton effect of ribonuclease observed at 278 mμ and at neutral pH should be assigned to the buried tyrosyl residues which stabilize the tertiary structure of the enzyme. In addition, however, a free tyrosyl residue generates a Cotton effect when ionized. The Cotton effect observed at pH 11.5, where only the free residues are ionized (Shugar, 1952), seems to be the sum of both of these. The superimposition of these two Cotton effects apparently produces the bathochromic shift in the midpoint of the side-chain Cotton effect at pH 11.5 previously observed (Glazer and Simmons, 1965b).

Experimental Section

Lyophilized ribonuclease A, isolated by chromatography and free of phosphate, was obtained from the Worthington Biochemical Corp., Freehold, N. J. *N*-Acetylimidazole (Aceto Chemical Corp.) was stored over phosphorous pentoxide *in vacuo* after recrystallization from dry benzene or isopropenyl acetate. Urea was recrystallized from 95% ethanol and air dried, and solutions were prepared immediately prior to use. All other chemicals were reagent grade.

Ribonuclease concentrations were determined spectrophotometrically based upon a molar absorptivity of $\epsilon_{277.5}$ 9.8×10^3 M^{-1} cm^{-1} (Sela and Anfinsen, 1957), and a molecular weight of 13,683 (Hirs *et al.*, 1956). Concentrations of the acetylated enzymes were determined following deacetylation with 1 M hydroxylamine (*vide infra*).

Acetylation of ribonuclease was carried out by addition of an 180-fold molar excess of dry *N*-acetylimidazole to 4.5 mg of ribonuclease/ml either of 0.02 M sodium Veronal or 0.02 M sodium Veronal plus 8 M urea, pH 7.5, 23°. The reaction was allowed to continue for 45 min. Solutions were then dialyzed in pretreated Visking cellulose dialysis tubing (Callanan *et al.*, 1957) against large excess of 0.1 M phosphate buffer, pH 6.1 or 7.0, 4°, for three changes of at least 8 hr each.

The degree of modification of tyrosyl residues was determined by measurements of spectra based on $\Delta\epsilon_{278}$ 1160/mole of tyrosyl residues acetylated (Simpson *et al.*, 1963). Modification of amino groups was assayed using an automated ninhydrin method. Samples of 0.1 ml, 4 mg/ml, were diluted into 0.9 ml of 0.2 M citrate buffer at pH 5.0, sampled at 0.42 ml/min and mixed with ninhydrin reagent (Lenard *et al.*, 1965) at a flow rate of 1.2 ml/min. Color was developed at 100° for 15 min and quantitated photometrically at 550 mμ in an Auto-Analyzer system (Technicon Instrument Corp., Chauncey, N. Y.), using phenylalanine as a standard.

Deacetylation of the modified proteins was performed either with 1 M hydroxylamine at 25° in pH 7.5, 0.1 M phosphate buffer for 10 min, or alternatively, with 0.1 M hydroxylamine for 1 hr in the same buffer at 25°. Labile acetyl groups were determined as previously described (Simpson *et al.*, 1963) except that 0.1 M phosphate buffer, pH 7.5, was employed. Ribonuclease activity was determined with soluble yeast ribonucleic acid as a substrate as described by Anfinsen *et al.* (1954). Assays were performed at 25° with 5–10 μg of ribonuclease/ml of 0.1 M sodium acetate buffer, pH 5.

pH was determined with a Radiometer pH meter using a general-purpose glass electrode. Absorbance measurements at discrete wavelengths were determined with a Zeiss PMQII spectrophotometer, while continuous absorption spectra were obtained with either a Cary Model 11 or Model 15 automatic recording spectrophotometer.

Optical rotatory dispersion measurements were performed in a Cary Model 60 automatic recording spectropolarimeter at 25°. Generally, measurements were performed with a 5-mm cell and protein concentration of about 0.4% from 350 to 250 mμ, with a 0.5-mm cell and the same protein concentration from 220 to 280 mμ, and with a 0.5-mm cell and a protein concentration of 0.08% from 200 to 225 mμ. The slit width of the instrument was programmed to yield constant light intensities at all wavelengths. In areas of high absorbance, absolute values for specific rotation were confirmed at two or more protein concentrations or path lengths, eliminating the possibility of spurious Cotton effects (Urnes and Doty, 1961). The data are expressed as specific rotation in degrees and are not corrected for the refractive index of the solvents employed. Circular dichroism measurements were performed with a Durrum–Jasco spectropolarimeter with a circular dichroism attachment. Conditions employed were identical with those used for the rotatory dispersion measurements. The results are expressed as the observed ΔA_{L-R} for a 0.4% solution of ribonuclease in a 5-mm cell.

Results

The ultraviolet absorption spectra of native ribonuclease and ribonuclease acetylated both in the presence and the absence of 8 M urea are shown in Figure 1. The hypo- and hypsochromic shift in the absorption of tyrosyl residues, previously demonstrated, consequent to acetylation of the phenolic hydroxyl group is apparent (Schlögl *et al.*, 1953; Simpson *et al.*, 1963). Upon acetylation in aqueous solution, the absorption maximum is shifted from 277.5 for the native enzyme to 279 mμ, and the absorptivity at 278 mμ is markedly decreased. The residual spectral properties of this modified protein in the region of 280 mμ largely reflect the absorption of the remaining unmodified tyrosyl residues. Based on the change of molar absorptivity at 278 mμ, $\Delta\epsilon_{278}$ 1160, for the conversion of *N*-acetyltyrosine to *N*,*O*-diacetyltyrosine, three tyrosyl residues are modified in this instance, in accord with previous findings (Riordan *et al.*, 1965a). This derivative will be referred to as Ac_3RNAase. Ribonuclease (RNAase) acetylated in the presence of 8 M urea absorbs maximally at approximately 262 mμ (Figure 1). The magnitude of the decrease in absorptivity at 278 mμ demon-

TABLE I: Characteristics of Modified Ribonucleases.[a]

Sample	Act. (% control)	Hydroxamates (mole/mole)	*O*-Acetyltyrosyls (mole/mole)	Amino Groups (% control)
Native RNAase	100	0	0	100
Ac_3RNAase	85	3.0	3.0	21
Ac_6RNAase	<5	5.9	6.0	22
Deacylated[b] Ac_6RNAase	65	0	0	22

[a] Samples were acetylated as described in Experimental Section. Hydroxamates were determined at pH 7.5. *O*-Acetyltyrosyl residues were determined spectrophotometrically. Amino groups were determined by ninhydrin color. [b] Ac_6RNAase was deacylated with 1 M hydroxylamine for 10 min at pH 7.5, 25°.

strates that all six tyrosyl residues of ribonuclease are acetylated when the reaction is performed in urea. This derivative is designated Ac_6RNAase. The number of residues modified in these two derivatives, as determined by acethydroxamate formation, is identical with that measured by spectrophotometry (Table I).

Ac_3RNAase is 85% as active as the native enzyme, but Ac_6RNAase exhibits only between 0 and 5% of the control activity after dialysis against aqueous buffers to remove urea (Table I). As previously noted, *N*-acetylimidazole also interacts with amino groups of proteins, although less readily than acetic anhydride in most instances examined (Riordan *et al.*, 1965a). However, about 75–80% of the ninhydrin-reactive groups of ribonuclease are modified on acetylation with acetylimidazole either in the presence or absence of urea (Table I). This greater degree of lysine modification is most probably related to the large excess of *N*-acetylimidazole required to acylate the free tyrosyls of ribonuclease, larger than that needed for many other proteins (Riordan *et al.*, 1965a).

These results are consistent with the possibility either that acetylation of buried tyrosyl residues might cause the loss of activity or that the lysyl residue at position 41 (Hirs *et al.*, 1960), known to be essential for activity (Hirs *et al.*, 1961), might be masked in aqueous solutions but become accessible to *N*-acetylimidazole in urea, thereby accounting for the inactivation. These alternatives can be examined since hydroxylamine should deacetylate *O*-acetyltyrosyl but not *N*-acetyllysyl residues.

Exposure of Ac_6RNAase to 0.1 M hydroxylamine at pH 7.5 in 0.1 M phosphate buffer progressively and continuously increases the absorbance at 278 mμ until, after 40 min of incubation at 25°, it has returned to that characteristic of native ribonuclease, denoting deacylation of all six tyrosyl residues. Further, deacylation increases enzymatic activity from 0–5 to 50–65% of the value observed for the native enzyme (Table I). These results are consistent with the hypothesis that buried tyrosyl residues are essential to the maintenance of an enzymatically effective ribonuclease structure. Their acetylation might prevent the refolding of the molecule into an active conformation which would

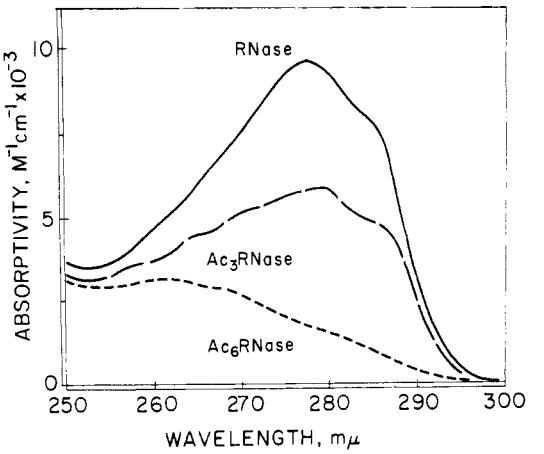

FIGURE 1: Ultraviolet absorption spectra of native (———) ribonuclease, and ribonuclease acetylated with a 180-fold molar excess of *N*-acetylimidazole in the absence (— —) and the presence (----) of 8 M urea. Spectra were determined in 0.1 M phosphate buffer, pH 6.1, following dialysis to remove denaturing agents and modifying reagents.

be expected to occur upon removal of urea. Deacylation, however, would then allow refolding to take place, resulting in restoration of the active three-dimensional structure.

Comparison of the optical rotatory dispersion curves of the native enzyme, of the acetylribonucleases, and of their deacetylated products support this interpretation (Figure 2). Native ribonuclease exhibits a large, negative Cotton effect centered at approximately 278 mμ. The intrinsic Cotton effect of the protein has a trough at 228 mμ and crosses over to positive rotation at 215 mμ. Urea (8 M) renders plain the rotation of ribonuclease through the aromatic absorption band and decreases the levorotation at the 228-mμ trough (*vide infra*). Following removal of urea, the optical rotatory dispersion of the resultant, refolded protein is identical with that of the native enzyme in aqueous solution.

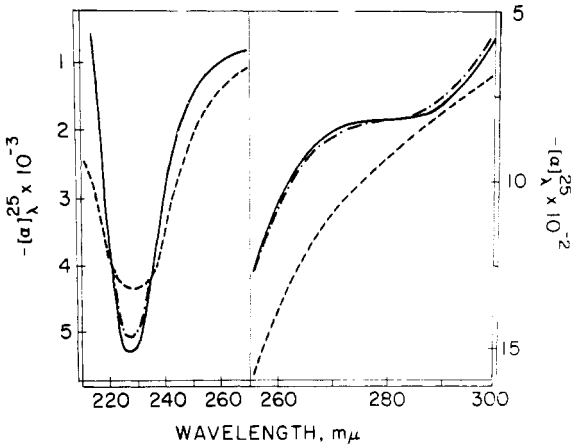

FIGURE 2: Optical rotatory dispersion of native ribonuclease (———), Ac_3RNAase (———), Ac_6RNAase (— —), and Ac_6RNAase following deacetylation with 1 M hydroxylamine as under Experimental Section (—·—).

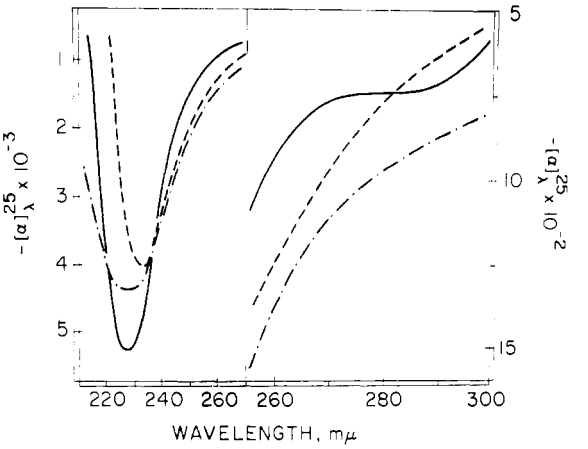

FIGURE 3: Optical rotatory dispersion of native ribonuclease in 45% dioxane (———), 1% sodium dodecyl sulfate (--------), and 8 M urea (—·—). The dioxane and sodium dodecyl sulfate samples were contained in 0.05 M phosphate buffer, pH 6.1, while the urea solution was unbuffered.

When compared to the native enzyme at wavelengths longer than 215 mμ, the optical rotatory dispersion of Ac_3RNAase is unaltered. Notably, both the side-chain Cotton effect centered at 278 mμ and the trough at 228 mμ are identical with those of native ribonuclease (Figure 2).

In contrast, the optical rotatory dispersion of Ac_6-RNAase differs markedly from that of native ribonuclease. The Cotton effect in the region of the tyrosyl absorption maximum is abolished. In the region of the trough of the intrinsic Cotton effect the rotation is less negative and the crossover to positive rotation is displaced from 215 to 206 mμ (Figure 2). In fact, the optical rotatory dispersion of Ac_6RNAase in aqueous solutions is quite similar to that of the native enzyme in 8 M urea. However, deacetylation of Ac_6RNAase with hydroxylamine restores both the rotation in the region of 260–300 mμ and that at shorter wavelengths to resemble again that of the native enzyme (Figure 2).

Since acetylation of the three free tyrosyl residues does not alter the optical rotatory dispersion of ribonuclease, these residues apparently do not generate the Cotton effect at 278 mμ. Therefore, the possible role of the three buried residues was evaluated further in various solvent systems. It has been reported that 1% sodium dodecyl sulfate exposes *one* of the buried tyrosyl residues of native ribonuclease (Bigelow and Sonenberg, 1962) and completely abolishes the 278-mμ Cotton effect (Glazer and Simmons, 1965b), and our observations agree entirely with these data (Figure 3). Similarly, 8 M urea, known to normalize the spectral titration curve of ribonuclease by exposing all of the buried tyrosyl residues (Sela and Anfinsen, 1957), also abolishes the Cotton effect at the aromatic absorption band (Figure 3). In contrast, 45% dioxane neither exposes any of the buried tyrosyl residues (Bigelow and Krenitsky, 1961) nor abolishes the Cotton effect at 278 mμ (Figure 3).

The optical rotatory dispersion of ribonuclease at varying pH values between 6 and 11.5 was also examined (Figure 4). At pH 6, the Cotton effect at 278 mμ is symmetric and smooth, but at higher pH values it becomes asymmetric and complex. Moreover, at pH 11.5, the midpoint of the Cotton effect shifts to longer wavelengths, apparently intermediate between the absorption maxima for ionized and un-ionized tyrosyl residues. The *difference dispersion* between the rotatory dispersion observed at neutral and at alkaline pH is a smooth curve centered at approximately 294 mμ, the absorption maximum for the phenolate of tyrosine (Figure 4 inset).

Since the peptide backbone contributes rotational components which interfere with the precise assignment of the midpoint(s) of Cotton effects in the 280-mμ region, measurements of circular dichroism were performed. At pH 6.1 native ribonuclease exhibits a negative, dichroic band with a maximum at 278 mμ, and also a positive band at shorter wavelengths (Figure 5). At pH 11.5, however, the maximum of the ellipticity band is shifted to 289 mμ (Figure 5). Further, the shorter wavelength band also seems to undergo a shift to longer wavelengths. This maximum at 289 mμ does not correspond to the absorption maximum either of an ionized or of an un-ionized tyrosyl residue. It apparently represents the sum of two optically active absorbing species of tyrosine, one due to ionized free residues and another due to un-ionized, buried residues. Subtraction of the ellipticity bands observed at pH 6.1 and 11.5 yields a *difference dichroism curve* which is a symmetric, negative band with a maximum at 294 mμ, precisely the wavelength of maximum absorption of ionized tyrosyl residues (Figure 5).

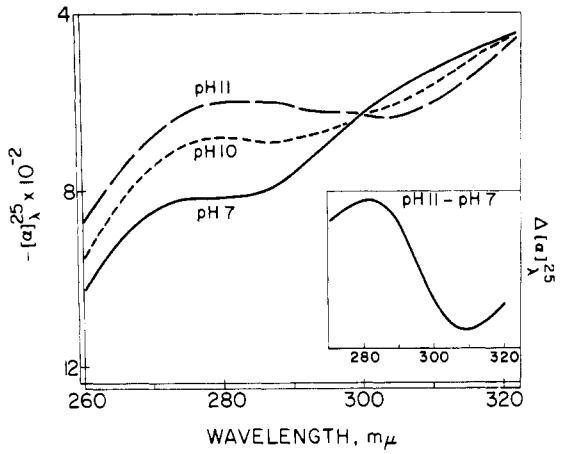

FIGURE 4: Optical rotatory dispersion of native ribonuclease at varying pH values, as indicated. A sample of ribonuclease (4 mg/ml) in 0.01 M phosphate, pH 6, was successively adjusted to pH 7, 10, and 11 with 6 N sodium hydroxide, and the rotatory dispersion determined. The rotations were corrected for the dilutions due to addition of alkali. The rotation at pH 6 is identical with that shown for pH 7, and that at pH 11.5 with that shown for pH 11. The inset is the difference dispersion between pH 7 and 11.

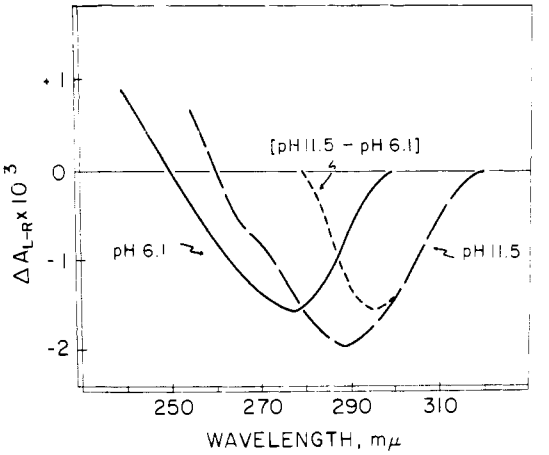

FIGURE 5: Circular dichroism of native ribonuclease at pH 6.1 (———) in 0.1 M phosphate and at pH 11.5 (— —) in 0.2 M glycine sodium hydroxide. The difference dichroism curve (- - - -) is derived by subtraction of the bands observed at the neutral and alkaline pH values.

Discussion

Operationally, the Cotton effects of proteins may be considered to be of three types. *Intrinsic Cotton effects* (Blout *et al.*, 1962) arise from rotational asymmetry of the chromophores of the polypeptide backbone and are sensitive to alterations in the secondary and tertiary structure of proteins or polypeptides. *Extrinsic Cotton effects* (Ulmer and Vallee, 1965) are superimposed upon the plain rotatory dispersion of proteins themselves. They are due to the asymmetric interaction of agents such as prosthetic groups, coenzymes, substrates, inhibitors, or metal atoms with proteins or polypeptides and have been employed to investigate the stereospecific interaction of these molecules or atoms, particularly with functional proteins. Finally, amino acid side chains may give rise to *side-chain Cotton effects* owing to their asymmetric environment in the highly structured conformation of proteins.

Potentially, side-chain Cotton effects in the 250- to 300-mμ spectral range might arise from hindered rotation about the α-carbon of cystinyl, phenylalanyl, tryptophanyl, or tyrosyl residues. The Cotton effects due to cystine in proteins would be expected to occur in the wavelength range from 250 to 270 mμ (Beychok, 1965), as would those due to phenylalanine (Moscowitz *et al.*, 1965). Asymmetry in the microscopic environment of tyrosine and tryptophan might be expected to result in Cotton effects at wavelengths longer than 270 mμ.

Although in most instances the precise basis of side-chain Cotton effects of proteins is ambiguous, at present the effect of selective chemical modification of suspected residues and the influence of pH and solvent variation on the side-chain Cotton effects of proteins might permit their assignment to the underlying chromophoric system, as has been possible in insulin (Beychok, 1965). Thus, the identification of side-chain Cotton effects generated by tyrosyl residues of proteins, *e.g.*, ribonuclease, should extend the scope of spectrophotometric titrations (Shugar, 1952) and perturbation spectroscopy (Laskowski, 1966), until now the physical approaches most widely used in delineation of the role of tyrosine in the structure and function of proteins.

Such Cotton effects due to tyrosyl residues are the primary subject of this communication. Bovine pancreatic ribonuclease was chosen for study since it seemed particularly suitable for the evaluation of the relative contributions of free and buried tyrosyl residues to side-chain Cotton effects. Since the enzyme does not contain tryptophan, one of the residues which might originate side-chain Cotton effects, a potential source of difficulty in interpretation is eliminated. The locations of the tyrosyl residues in the primary structure are known (Hirs *et al.*, 1961; Smyth *et al.*, 1962; Potts *et al.*, 1962; Spackman *et al.*, 1960). Spectrophotometric titration (Shugar, 1952), perturbation spectroscopy (Laskowski, 1966), iodination (Cha and Scheraga, 1962; Donovan, 1963), and *O*-acetylation with *N*-acetylimidazole (Riordan *et al.*, 1965a) demonstrate that three tyrosyl residues are free at the surface of the molecule and three are buried within the interior. *N*-Acetylimidazole, a selective acetylating agent, modifies the free tyrosyl residues of native proteins (Simpson *et al.*, 1963; Riordan *et al.*, 1965a). *O*-Acetylation with this agent of reversibly denatured proteins also enables investigation of the role of buried tyrosyl residues in the formation and maintenance of protein

structure, as exemplified by studies of trypsin (Riordan *et al.*, 1965b).

The intrinsic Cotton effect of ribonuclease is typical neither of a completely random nor a completely helical polypeptide structure (Blout *et al.*, 1962) and its meaning has not as yet been assigned. The potential contributions of a Cotton effect associated with the 220-mμ absorption band of tyrosine (Malik, 1961) complicate interpretation, especially since ribonuclease has a large negative Cotton effect near the 277.5-mμ absorption maximum (Beychok, 1965; Glazer and Simmons, 1965b; Cathou *et al.*, 1965) (see also Figure 2). The sign of this Cotton effect is opposite to that observed either for free tyrosine (Hooker and Tanford, 1964) or for poly-L-tyrosine (Fasman *et al.*, 1964). Its approximate magnitude can be calculated by subtracting a plain dispersion curve which intersects the observed curve at 278 mμ. Expressed as specific rotation it is about 80°, equivalent to 11,000° of molar rotation, *i.e.*, markedly greater than the Cotton effects either of the free amino acid or of the homopolymer.

Acetylation of native ribonuclease with *N*-acetylimidazole modifies three tyrosyl residues. This derivative is enzymatically nearly as active as the native enzyme (Table I). Similarly, diiodination of three tyrosyl residues does not alter ribonuclease activity (Donovan, 1963). Apparently, these free tyrosyl residues do not play a critical role either in activity or in maintenance of the functional structure of the enzyme. Although *N*-acetylimidazole modifies the amino groups of ribonuclease to a greater degree than those of many other proteins and polymers examined (Riordan *et al.*, 1965a), the functionally critical lysyl residue at position 41 (Hirs *et al.*, 1960, 1961) is apparently not modified when the native protein is acetylated, since activity is not changed markedly (Table I). The variable facility in modification of this lysyl residue dependent on the agent employed has been established previously (Klee and Richards, 1957; Cooke *et al.*, 1963).

While the present experiments were not designed to demonstrate the identity of the three *O*-acetylated tyrosyls to the three tyrosyls shown to be free by iodination and sequence studies (Cha and Scheraga, 1962; Donovan, 1963) some of the spectral data suggest that this may well be the case. *O*-Acetylation shifts the absorption maximum of *N*-acetyltyrosine from 275 to 262 mμ, and markedly decreases the coefficient of absorptivity (Schlögl *et al.*, 1953; Simpson *et al.*, 1963). On this basis, the residual spectral properies of Ac_3RNAase in the region of 280 mμ largely reflect the absorption of the remaining, unmodified tyrosyl residues. Native ribonuclease absorbs maximally at 277.5 with a coefficient of absorptivity of 9.8×10^3 M^{-1} cm^{-1}. As expected, the coefficient of absorptivity of Ac_3RNAase is lower, 6.2×10^3 M^{-1} cm^{-1}, but the maximum absorption of this modified protein is shifted to 279 mμ (Figure 2). Similarly, the spectral maximum of free tyrosine at 275-mμ shifts to longer wavelengths, *i.e.*, 277.5 mμ, when incorporated into ribonuclease. This bathochromic shift has been interpreted to reflect the "burying" of some of the tyrosyl residues within an hydrophobic environment (Bigelow, 1961), and such a bathochromic shift of the residual tyrosyl absorption in Ac_3RNAase is consistent with the assumption that the residues generating it are buried in the interior of the folded molecule.

Acetylation in aqueous solution does not alter the trough of the intrinsic Cotton effect of ribonuclease near 228 mμ, suggesting that acetylation has not engendered major conformational changes. Moreover, acylation of these three surface tyrosyl residues does not change the side-chain Cotton effect of ribonuclease at 278 mμ, although the modification markedly alters the absorption spectrum. If the surface residues did give rise to this Cotton effect, the midpoint of the anomalous dispersion of Ac_3RNAase would be expected to be shifted to 262 mμ, the absorption maximum of *O*-acetyltyrosine. The failure to observe such a shift militates against the hypothesis that this side-chain Cotton effect should be attributed to the residues modified by acetylation in aqueous solutions. Instead, such data imply that the side-chain Cotton effect might be due to asymmetry in the environment of one or more of the buried tyrosyl residues.

When acetylation is performed in urea, all six tyrosyl residues are modified and enzymatic activity is virtually abolished, in marked contrast to the slight effect of *O*-acetylation of the three free tyrosyls. This loss of activity could be due to either acetylation of the functionally critical lysyl residue at position 41 (Hirs *et al.*, 1960, 1961) or to prevention of refolding of the molecule to the active conformation upon removal of urea. Acetylation in urea does not modify lysine 41 significantly, since treatment with hydroxylamine largely restores the enzymatic activity of Ac_6RNAase (Table I). As employed here, hydroxylamine only deacylates *O*-acetyltyrosyl residues, and not *N*-acetyllysyl residues.

A number of separate lines of evidence support the view that acetylation of the buried tyrosyl residues prevents refolding and restoration of the native, active conformation, on removal of urea. Hydroxylamine deacylates all six tyrosyl residues at the same first-order rate. If, subsequent to removal of urea, Ac_6-RNAase would regain a significant degree of organized tertiary structure, the buried and free tyrosyls would be expected to deacylate at different rates. Compared with the native enzyme, the optical rotatory dispersion characteristics of Ac_6RNAase, and its decreased solubility above pH 6.5, confirm that it has, indeed, undergone a loss of secondary and tertiary structure (Figure 2). The 278-mμ Cotton effect is abolished, and the far-ultraviolet dispersion pattern is altered (Figure 2). The rotatory dispersion of Ac_6RNAase in aqueous solution is very similar, in fact, to that of the native enzyme when unfolded in 8 M urea.

Based on the results of titration and spectral studies the buried tyrosyl residues have been thought to stabilize the native conformation of ribonuclease, either through hydrophobic interactions or through formation of hydrogen bonds with aspartyl carboxyl groups (Shugar, 1952; Scheraga, 1960; Hermans and Scheraga, 1961; Scheraga and Rupley, 1962). The solubility of phenol

and acetylphenol in aqueous and nonpolar solvents would suggest that little difference in the hydrophobicity of acetylated and unmodified tyrosyl residues should be expected. Acetylation might block refolding by preventing hydrogen bonding to another group, or simply by steric hindrance.

The failure of Ac_6RNAase to refold spontaneously into an enzymatically active conformation on removal of urea is quite analogous to similar behavior of acetyltrypsin (Riordan *et al.*, 1965b). The present results are consistent with the long-postulated role of buried tyrosyl residues in the maintenance of the native structure of ribonuclease (Scheraga and Rupley, 1962). In contrast, modification of lysyl or carboxyl groups of the native enzyme (Epstein *et al.*, 1963) does not interfere with refolding of ribonuclease, even after disulfide reduction.

The Cotton effect at 278 mμ reflects the effect of protein conformation on the asymmetric environment of buried tyrosyl residues. Solvent systems which expose buried tyrosyl residues, *e.g.*, urea, also abolish the side-chain Cotton effect, while 45% dioxane neither exposes buried residues nor alters the anomalous rotatory dispersion (Figure 4). One per cent sodium dodecyl sulfate abolishes the anomalous optical rotatory dispersion of ribonuclease (Glazer and Simmons, 1965b) (Figure 4). At this concentration of the detergent, only one tyrosyl residue, buried in aqueous media, becomes exposed (Bigelow and Sonenberg, 1962). In conjunction with the present data, this observation would suggest that the Cotton effect at 278 mμ might be assigned to this particular residue, "B" in the terminology of Bigelow (1961). Unfortunately, the *O*-acetyltyrosyl bond is labile at alkaline pH; hence, the combination of acetylation and OH^- concentration on the side-chain Cotton effect is not tested readily.

The apparent midpoint of the side-chain Cotton effect shifts to longer wavelengths at pH 11.5, where only the three surface tyrosyl residues are ionized, an observation consistent with the suggestion that this Cotton effect might arise from the surface tyrosyl residues of the protein (Glazer and Simmons, 1965b). However, such data are also consistent with a superimposition of the Cotton effect at 278 mμ and pH 7.0, previously noted to be due to buried residues, on a Cotton effect centered at 294 mμ and *newly generated* by ionization of a free residue(s) at pH 11.5. Both these Cotton effects might be present at alkaline pH but not be resolved. Such alternatives could not be decided, however, by rotatory dispersion studies alone. Hence, circular dichroism was measured to examine this proposition. At neutral pH the maximum ellipticity of the dichroic band coincides with the absorption maximum of the tyrosyl residues. In contrast, at pH 11.5 the maximum is found at 289 mμ, intermediate between the maxima for ionized and un-ionized tyrosyl residues. The symmetric, dichroic difference band is centered at 294 mμ, the absorption maximum of ionized tyrosyl residues (Figure 5).

Thus, at pH 11.5, the side-chain Cotton effect in the region of tyrosyl absorption of ribonuclease appears to involve two separate Cotton effects, arising both from a buried tyrosyl residue(s), the only one observed at neutral pH, and from a free tyrosyl residue(s) whose microscopic environment is rendered asymmetric on ionization of the phenolic hydroxyl group at alkaline pH. At pH 11.5, the observed Cotton effect is the unresolved sum of both. The new asymmetric absorption band at alkaline pH might arise from a specific interaction of an ionized surface tyrosyl with another protein residue, or might indicate an alteration of protein conformation, a suggestion which seems less likely since rotation at the trough of the intrinsic Cotton effect is not markedly changed at the higher pH (Glazer and Simmons, 1965b).

The present data further support the hypothesis that the primary sequence determines the secondary and tertiary structure of proteins as discussed by Anfinsen (1962). Both ribonuclease and trypsin (Riordan *et al.*, 1965b) readily unfold in urea and refold on its removal, or when the primary sequence altered by acetylation is reversed by deacylation of the modified tyrosyls with hydroxylamine. Thus, acetylation of the buried tyrosyl residues of either protein apparently prevents refolding to the native, active conformation. Studies such as these, utilizing *O*-acetylation of tyrosyl residues in the native and denatured states and its reversal (Riordan *et al.*, 1965b) while allowing further exploration of the role of buried tyrosyl residues in the formation of the tertiary structure of proteins, will also likely elucidate the origin of side-chain Cotton effects in the region of the spectrum where aromatic amino acids absorb radiation. In this regard, preliminary results of other chemical modifications of the tyrosyl residues of ribonuclease are consistent with thoses obtained with *N*-acetylimidazole, and hence with the interpretation of the origin of the side-chain Cotton effects.

Acknowledgment

The authors are indebted to Dr. Leonard Katzin, Argonne National Laboratories, Argonne, Ill., for his generous cooperation in obtaining the circular dichroism data.

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