Characteristic Extrinsic Cotton Effects of Azo Proteins*

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ABSTRACT: Diazonium salts are known to react readily with various amino acid side chains in proteins to form covalently bound chromophoric derivatives which absorb radiation between 300 and 600 mμ. We now have found that such protein azo chromophores can be optically active, resulting in distinctive, extrinsic Cotton effects. Mild treatment of a variety of proteins with diazotized *p*-arsanilic acid, modifying only a fraction of the total number of the potentially reactive residues present, generates circular dichroic bands at wavelengths above 300 mμ. These cluster generally within three spectral intervals: 320 to 340, 375 to 395, and 420 to 460 mμ. However, variations in the number, position, sign, and magnitude of the bands comprising each spectrum combine to make the optical activity between 300 and 600 mμ seemingly unique for each azo protein studied. Significantly, the circular dichroic spectra are distinctive even though the associated absorption spectra are not. The absorption spectra of the azo proteins, generated by modification of a single protein, *e.g.*, ribonuclease, with different diazonium salts, *i.e.*, those derived from *p*-aminobenzoic acid, *p*-arsanilic acid, 5-amino-1*H*-tetrazole, and 5-aminoindazole, are all similar as are the corresponding circular dichroic spectra. Each azoribonuclease exhibits a negative circular dichroic band at 380 mμ, a positive band at 450 mμ and no detectable optically active band in the 325-mμ region of the azo chromophore absorption. The dichroic spectra differ from one another only in their molecular ellipticities. Azoovalbumins, generated in a like manner, have absorption spectra similar to those of the azoribonucleases, but their circular dichroic spectra, while similar to one another, differ distinctly from those of the azoribonucleases. The azoovalbumins are optically active in the region of maximum absorption of the azo chromophores near 325 mμ.

In addition, two dichroic bands are located at longer wavelengths, similar to those of the azoribonucleases but of opposite signs. Denaturation with guanidine hydrochloride destroys the distinctive differences in the circular dichroic spectra of the azo proteins. The data indicate that the extrinsic Cotton effects, generated by covalent modification of proteins with diazonium salts, can reflect differences as well as monitor changes in structure and conformation, characteristic of different proteins.

It is now well known that the molecular conformation of proteins can critically influence the reactivity of their amino acid side chains toward chemical reagents. Chemical modifications of *native* proteins generally affect only a few of the total number of side chains potentially available for reaction, whereas in denatured proteins virtually all such residues can be modified. Hence, the native conformation renders a proportion of the residues unreactive. Further, particular residues, especially those at active sites of native enzymes, may display greatly enhanced reactivity toward specific organic agents as compared to that of randomly oriented proteins, amino acids, or polypeptides. Clearly, conformation can endow some residues with unusual reactivities. At the present juncture, however, there are no specific factors known which might explain such variable reactivities of amino acid side chains. Moreover, the methods which might elicit such information are limited in both scope and number.

The chromophoric properties of the derivatives formed upon the coupling of diazonium salts with various amino acid side chains long have attracted attention to this class of chemical reagents. The characteristics of the absorption spectra of azo derivatives of tyrosine, histidine, and lysine have seemed sufficiently distinctive to allow the direct qualitative and quantitative identification of the corresponding modified residues in proteins, based on these spectra. However, in practice, the results have proved disappointing; azo proteins display multiple broad spectral bands whose overlap frequently cannot be resolved with the required precision. Hence, these absorption spectra have been of limited value for investigations of structure–function relationships. Significantly, modifications with diazonium salts under mild conditions are known to affect but a *few* of the total number of tyrosyl, histidyl, and lysyl residues potentially modifiable in proteins, suggesting that such modifications might well depend upon features of three-dimensional structure.

While proteins exhibit optical activity characteristic of their macromolecular conformation, as reflected in "intrinsic" Cotton effects, their interaction with chromophoric molecules such as coenzymes, substrates, inhibitors, or metal atoms at specific protein sites can result in "extrinsic" Cotton effects. Such Cotton effects reflect structural features of the specific combining sites and their environments (Ulmer and Vallee, 1965). In addition, in a few instances, chemical modifications of protein side chains resulting in chromophoric derivatives have also been found to generate extrinsic Cotton effects (Dowben and Orkin, 1967; Beaven and Gratzer, 1968; Johnson *et al.*, 1968; Meloun *et al.*, 1968; Kagan and Vallee, 1969; Sigman *et al.*, 1969). The azo chromophores of arsanil-

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azocarboxypeptidase are not only optically active but also environmentally sensitive (Kagan and Vallee, 1969), thus suggesting the suitability of combining modifications with diazonium salts and measurements of circular dichroism for probing of protein structure. Toward this end, we now have coupled a number of proteins with various diazonium salts under mild conditions, modifying a limited number of residues. Such reactions, in general, generate conformation-dependent extrinsic Cotton effects which appear to be remarkably distinctive for particular pairs of proteins and diazonium salts. Notably, the circular dichroic spectra of different proteins, coupled with the same diazonium salt, differ markedly, even when the absorption spectra do not. While only a limited number of proteins have been studied, the data on these specific systems suggest that they reflect a general phenomenon. A preliminary account of these studies has appeared (Fairclough and Vallee, 1969).

Materials and Methods

p-Arsanilic acid and *p*-aminobenzoic acid were obtained from Eastman, and 5-amino-1*H*-tetrazole monohydrate and 5-aminoindazole from Aldrich. Poly-L-tyrosine (lot P-2081, mol wt 40,000–100,000) was purchased from Cyclo Chemical. Guanidine hydrochloride, Ultra Pure grade, was a product of Mann Research Laboratories and Bio-Gel P-6, 50–100 mesh, of Bio-Rad Laboratories.

Ovalbumin, bovine carboxypeptidase A, bovine α-chymotrypsin, bovine ribonuclease A, and horse liver alcohol dehydrogenase were obtained from Worthington Biochemical Corp., bovine serum albumin and bovine γ-globulin from Pentex, Inc., porcine elastase from Schwarz BioResearch, Inc., rabbit muscle aldolase from Calbiochem, insulin from Lilly, apoconalbumin from Sigma, and nagarse from Biddle-Sawyer, N. Y. Alkaline phosphatase from *Escherichia coli* was prepared according to Simpson *et al.* (1968).

Diazonium Salt Preparation. The desired aromatic amine (0.5 mmole) was weighed and dissolved in 20 ml of 0.15 M HCl cooled to 0°. To this solution, 50 mg of sodium nitrite was added slowly with continuous stirring, and reaction was allowed to proceed for 15 min at 0°. These conditions were sufficient for maximal diazotization as determined by coupling the reagents with excess phenol. The pH of the solution was then increased to between 5.0 and 5.5 by the gradual addition of 3 M NaOH from a long-tipped syringe while the solution was stirred continually at 0°. The volume of the solution was adjusted to 25 ml to give a reagent concentration of 2×10^{-2} M. Suitable aliquots of the reagent mixture were used immediately for protein modification.

Sample Modification. Crystalline protein preparations were dissolved in 2.0 ml of 0.67 M sodium bicarbonate buffer (pH 8.8, 0°) just prior to modification. Those obtained as ammonium sulfate suspensions were dissolved by dialyzing against several 100-fold volume excesses of 0.67 M sodium bicarbonate buffer (pH 8.8, 0°). Protein concentrations were determined by measuring absorbance at 280 mμ. In most instances, chemical modification was carried out at a final protein concentration of 2×10^{-4} M; however, bovine γ-globulin, horse liver alcohol dehydrogenase, and *E. coli* alkaline phosphatase were modified at a concentration of 1×10^{-4} M, and rabbit muscle aldolase at 5×10^{-5} M.

The reaction was initiated by the addition of a suitable aliquot of the desired diazonium salt, prepared as above, to the protein solution, and the final volume was adjusted to 2.5 ml by addition of bicarbonate buffer. Coupling was allowed to proceed at 0° for the desired length of time and then terminated by addition to the reaction mixture of sufficient 0.1 M phenol to produce a final concentration equivalent to that of the diazonium salt. Each modified protein was separated from the reaction mixture and transferred into 0.10 M NaCl–0.05 M Tris buffer (pH 7.5) by gel filtration using a 0.9 × 55 cm Bio-Gel P-6 column. Coupling reactions with diazotized 5-aminoindazole were terminated by gel filtration at the desired time without prior phenol addition. In all cases the entire azo protein peak was collected, adjusted to a known volume, and the optical properties examined.

Because of its insolubility at pH 8.8, the poly-L-tyrosine polymer was modified at a more alkaline pH then employed for the proteins. The commercial polymer preparation was initially suspended in 0.5 M NaOH–0.5 M Na_2CO_3 and residual insoluble material removed by centrifugation. Then the polymer was precipitated twice by adjusting the pH to 8 and redissolved by suspension in 0.5 M NaOH–0.5 M Na_2CO_3 to form a stock polymer solution, approximately 0.02 N per tyrosyl residue. For modification the polymer, 1.1×10^{-2} N per tyrosyl residue, was treated with diazotized *p*-arsanilic acid (6.7×10^{-3} M) for 1 hr in 0.3 M NaOH–0.3 M Na_2CO_3 buffer (pH 12.5, 0°). Phenol was added to quench the reaction and the pH was adjusted to 11 with HCl. The smaller molecular weight components of the reaction mixture were removed by dialysis against sequential 500-fold volume excesses of 0.5 M NaCl. Since the polymer precipitates during this dialysis, it was twice redissolved by adjusting the pH to 10.5 as a precaution against the coprecipitation of noncovalently bound azo chromophores. The induction of helical structure in the polymer was carried out by dialysis at 40° against 0.01 M HCl as described by Fasman *et al.* (1964). The precipitated polymer was isolated by lyophilization. For optical studies samples were weighed out and the azotyrosyl content determined from the extinction coefficient of the appropriate model compounds (Tabachnick and Sobotka, 1959).

Experimental Measurements. A Radiometer Model 25 pH meter, fitted with a scale expander, using a type GK 2021C combination electrode was employed to measure pH. Absorption spectra were determined with a Cary 15 MS recording spectrophotometer. Circular dichroism measurements were performed with a Cary 60 recording spectrapolarimeter fitted with a Cary Model 6001 circular dichroism accessory as previously described (Kagan and Vallee, 1969). Slit widths were programmed to provide a constant band width of 15 Å. Calibration was checked using a 1-mg/ml solution of *d*-10-camphorsulfonic acid. Cells of 0.50-, 1.0-, and 2.0-cm path length were used for azo protein measurements. To ensure against artifacts in the circular dichroism data, the concentrations of azo proteins were chosen such that the maximum absorption of the azo chromophores did not exceed a level of 2.0. No optical activity was detected while scanning absorption bands of potassium dichromate solutions having comparable absorption intensities. The circular dichroism data for the azo proteins are expressed as the molecular ellipticity, $[\theta]$, in units of deg cm^2 per dmole of protein modified and those for the polymer of poly-L-tyrosine per dmole of tyrosine.

TABLE I: Azo Derivatives Produced upon Modification with Diazotized *p*-Arsanilic Acid.[a]

Protein	Moles/Mole of Protein	
	Monoazo-Tyr	Monoazo-His
α-Chymotrypsin	0.5	0.02
Elastase	1.1	0.1
Aldolase[b]	2.7	1.6
γ-Globulin	2.9	1.7

[a] Protein modification and sample preparation as described in Figure 1. [b] The behavior of the azo protein absorption spectrum upon change of pH varied from that expected for model compounds described previously and, hence, would seem to indicate the possibility of contributions from other, as yet uncharacterized, azo chromophores.

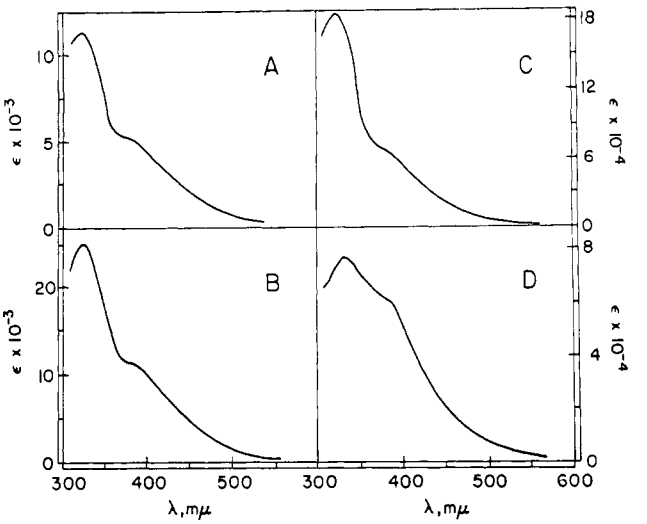

FIGURE 1: Absorption spectra of azo proteins in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). (A) Bovine α-chymotrypsin, (B) porcine elastase, (C) rabbit muscle aldolase, and (D) bovine γ-globulin. The proteins were treated with 2×10^{-3} M diazotized *p*-arsanilic acid for 30 min in 0.67 M bicarbonate buffer (pH 8.8, 0°) at concentrations of 2×10^{-4} M for α-chymotrypsin and elastase, 1×10^{-4} M for γ-globulin, and 5×10^{-5} M for aldolase. They then were separated from the reaction mixture and transferred into 0.10 M NaCl–0.05 M Tris buffer (pH 7.5) by gel filtration.

Results

Diazonium salts readily couple with proteins to form colored derivatives of tyrosyl, histidyl, and/or lysyl residues, among others. Modification of α-chymotrypsin, elastase, aldolase, and γ-globulin with 2×10^{-3} M diazotized *p*-arsanilic acid for 30 min at pH 8.8, 0°, generates the azo protein absorption spectra shown in Figure 1. These spectra, measured at pH 7.5, are all quite similar, with maxima near 325 mμ, shoulders between 380 and 400 mμ and broad envelopes extending to above 500 mμ, but they differ in intensities. Based on the molar absorptivities in alkali, proposed for the measurement of histidine and tyrosine (Tabachnick and Sobotka, 1960), formation of monazotyrosine predominates, though there are lesser degrees of histidyl modification (Table I). Under these conditions, a small number of residues are modified.

The similarity of the absorption spectra of these azo proteins is in marked contrast with the diversity of their circular dichroic spectra (Figure 2). Azo-α-chymotrypsin, in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5) displays two positive Cotton effects. One, located at 325 mμ, corresponds to the principal absorption band of azochymotrypsin; the other, at 450 mμ, is in a spectral region where individual absorption bands are not resolved clearly.

Azoelastase exhibits at least three Cotton effects. Two of these correspond closely in spectral position with those observed in modified α-chymotrypsin but are of opposite sign. The third, a positive Cotton effect, appears to correspond to the shoulder at 385 mμ in the absorption spectrum.

Modification of γ-globulin with diazotized *p*-arsanilic acid gives rise to a single, asymmetric, positive ellipticity band with a maximum near 375 mμ, likely reflecting transitions associated with the shoulder at 390 mμ in the absorption spectrum.

The circular dichroic spectrum of azoaldolase differs still further from the others shown. It exhibits two extrema: one, a positive ellipticity band, is located at 320 mμ, coincident with the absorption maximum at that wavelength; the second, a negative band at 395 mμ, may arise from an absorption shoulder at 390 mμ.

Noncovalent binding of azo compounds to these proteins does not account for the circular dichroic spectra observed here. Under the conditions used for modification, each protein was exposed to diazotized *p*-arsanilic acid which had been first coupled and inactivated with an equivalent amount of phenol. Following separation of the protein reaction mixture by gel filtration, optically active azo chromophores are not observed in the circular dichroic spectra of the protein products of this procedure.

Modification of these same proteins with double the amount of diazotized *p*-arsanilic acid (4×10^{-3} M) and for 1 hr rather than 30 min, increases the magnitude of some of the ellipticity values but does not bring about major changes

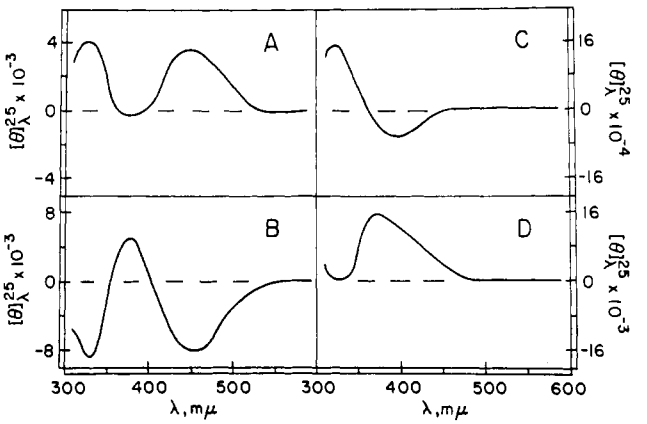

FIGURE 2: Circular dichroism of azo proteins in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). (A) α-Chymotrypsin, (B) elastase, (C) aldolase, and (D) γ-globulin. Protein modification and sample preparation as described in Figure 1.

TABLE II: Dichroic Bands Generated upon Modification with Diazotized *p*-Arsanilic Acid.[a]

Protein	λ_{max}	$([\theta] \times 10^{-3})$	λ_{max}	$([\theta] \times 10^{-3})$	λ_{max}	$([\theta] \times 10^{-3})$		
Alcohol dehydrogenase	325	(+48)	395	(−63)				
Aldolase	320	(+153)	395	(−55)				
Alkaline phosphatase	320	(−11)	385	(+6)	440	(−5)		
Carboxypeptidase	325	(−14)			435	(+15)	525	(−7)
Chymotrypsin	325	(+9)			450	(+6)		
Conalbumin (apo)	320	(−11)			420	(−7)		
Elastase	330	(−10)	380	(+6)	455	(−10)		
γ-Globulin			380	(+25)				
Insulin	340	(+9)			445	(−1)		
Nagarse	330	(+7)	390	(+5)				
Ovalbumin	320	(−2)	380	(+4)	445	(−6)		
Serum albumin					420	(+10)		
Ribonuclease			375	(−3)	445	(+11)		

[a] The proteins were treated with 4×10^{-3} M diazotized *p*-arsanilic acid in 0.67 M bicarbonate buffer at pH 8.8, 0°, for 1 hr. The optical properties were examined after the modified proteins were separated from the reaction mixtures and transferred into 0.10 M NaCl–0.05 M Tris buffer at pH 7.5, 25°, by gel filtration.

in the circular dichroic spectra (Table II). The modification of other single and multichain proteins under the same experimental conditions generates an additional variety of patterns of circular dichroic spectra (Table II). Again the extent of formation of azo derivatives appears to be low in these cases (Table III).

The circular dichroic bands which compose these spectra appear to be clustered primarily in three regions. Virtually all of the maxima of the ellipticity bands are distributed between 320 and 340 mμ, 375 and 395 mμ, or 420 and 455 mμ. These wavelength ranges are consistent with those of the known absorption maxima and shoulders of the common azo side-chain derivatives formed upon coupling with this reagent (Tabachnick and Sobotka, 1959) and are distinctive for each particular protein modified.

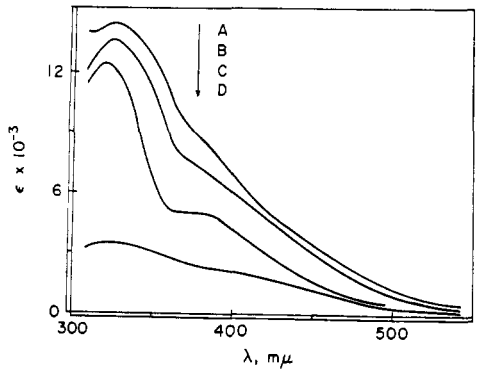

FIGURE 3: Absorption spectra of azoribonucleases in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). Modification was carried out for 30 min in 0.67 M bicarbonate buffer at pH 8.8, 0°, by exposure of 2×10^{-4} M ribonuclease to 2×10^{-3} M diazonium salt derived from either (A) *p*-aminobenzoic acid, (B) *p*-arsanilic acid, (C) 5-amino-1*H*-tetrazole, or (D) 5-aminoindazole. Samples were separated from the reaction mixture and transferred into 0.10 M NaCl–0.05 M Tris buffer (pH 7.5) by gel filtration.

Upon coupling with proteins, other diazonium salts can also generate optically active azo chromophores. For example, modification of ribonuclease (2×10^{-4} M) for 30 min at pH 8.8, 0°, with a 10-fold molar excess of the diazonium salt derived either from *p*-aminobenzoic acid, *p*-arsanilic acid, 5-amino-1*H*-tetrazole, or 5-aminoindazole produces the corresponding family of azoribonucleases which have closely related absorption spectra, differing largely in absorptivities

TABLE III: Azo Derivatives Produced upon Modification with Diazotized *p*-Arsanilic Acid.[a]

Protein	Monoazo-Tyr	Monoazo-His
Alcohol dehydrogenase[b]	1.0	0.7
Aldolase[b]	5.3	2.9
Alkaline phosphatase	0.8	0.5
Carboxypeptidase	2.4	0.2
Chymotrypsin	0.9	0.1
Conalbumin (apo)	1.3	0.7
Elastase	2.4	0.2
γ-Globulin	4.8	2.0
Insulin	1.2	0.5
Nagarse	0.8	0.1
Ovalbumin	0.9	0.3
Serum albumin	0.9	1.1
Ribonuclease	0.9	0.3

[a] Protein modification and sample preparation as described in Table II. [b] The behavior of the azo protein absorption spectra upon change of pH varied from that expected for model compounds described previously, and, hence, would seem to indicate the possibility of contributions from other, as yet uncharacterized, azo chromophores.

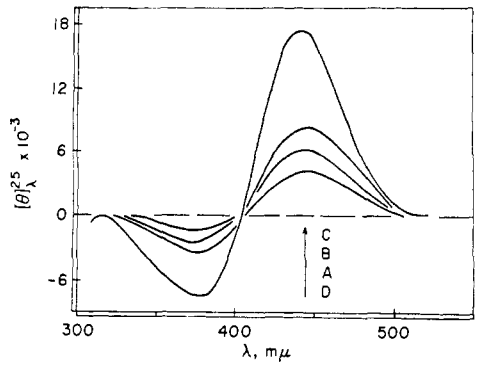

FIGURE 4: Circular dichroism of azoribonucleases in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). Protein modification, sample preparation and letter code as described in Figure 3.

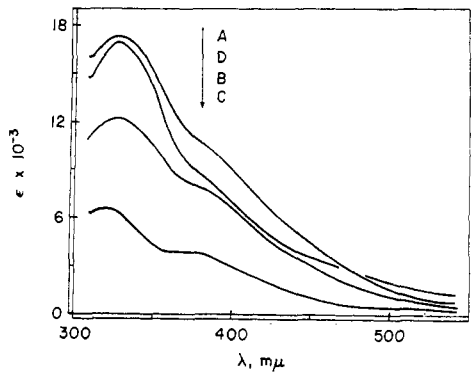

FIGURE 5: Absorption spectra of azoovalbumins in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). Protein modification, sample preparation and letter code as described for Figure 3.

but not greatly in other spectral details (Figure 3). While the magnitudes of the absorption spectra at pH 7.5 differ, all exhibit maxima between 320 and 330 mμ and shoulders in the region of 380 and 390 mμ, superimposed on broad bands extending beyond 500 mμ, features which are consistent with predominant tyrosyl modification (Tabachnick and Sobotka, 1959; Sokolovsky and Vallee, 1966) (Figure 3). Upon modification of ribonuclease with the diazonium salts of 5-aminoindazole, *p*-aminobenzoic acid, *p*-arsanilic acid, and 5-amino-1*H*-tetrazole, 0.15, 0.41, 0.50, and 0.57 residues of monazotyrosine are formed, respectively, while modifications of histidine are correspondingly less in each case, as calculated from the absorption spectra.

At pH 7.5, the circular dichroic spectra of the various azoribonucleases each display a negative dichroic band centered near 380 mμ corresponding to a shoulder in the absorption spectrum, and a positive dichroic band centered at 445 mμ, though the absorption spectra do not exhibit analogous, distinctive features (Figures 3 and 4). Although the locations of the circular dichroic bands of various azoribonucleases are identical one with the other, the order of their relative magnitudes differs from that of the corresponding absorption spectra. Thus, for example, the circular dichroic spectrum of ribonuclease modified with 5-diazonium-1*H*-tetrazole exhibits the largest ellipticity values, while that modified with *p*-diazoniumbenzoic acid has the most intense absorption spectrum (Figures 3 and 4).

Comparisons of such series of absorption and circular dichroic spectra for different azo proteins are even more revealing. Thus, when ovalbumin is coupled with the same series of diazonium reagents, the details of the resultant absorption spectra are similar to those of the corresponding azoribonucleases, although the relative order of the absorption intensities for the various derivatives differ (Figure 5). The circular dichroic spectra of the various azoovalbumins also resemble one another but differ distinctly from those of the azoribonucleases (Figure 6). In particular, the absorption maxima at 325 mμ are rendered optically active; for the ribonuclease derivatives this spectral region is optically inactive. More distinctly, each of the azoovalbumin derivatives exhibits a positive ellipticity band centered at about 385 mμ and a negative one near 445 mμ, similar in position but opposite in sign to those observed for the corresponding azoribonuclease derivatives. Moreover, the ovalbumin derivative formed upon modification with 5-diazoniumindazole has molecular ellipticity values of −25,000 and 40,000° for the 330- and 390-mμ bands, respectively, values greatly enhanced with respect to those of the other azoovalbumins.

The effects of conformational alterations on the circular dichroism of a particular azo chromophoric amino acid residue can be studied most unambiguously in a homopolymer of that amino acid capable of assuming distinctive and well-defined conformational states. Since azotyrosine derivatives are formed frequently in the proteins here examined, a poly-L-tyrosine polymer, containing 1 arsanilazotyrosyl chromophore/40 tyrosyl residues, was prepared to examine conformational contributions. Helical structure in the polymer was induced by the dialysis and heating procedure of Fasman *et al.* (1964), and the polymer was then redissolved in 0.10 M NaCl by adding NaOH until the pH was 10.2. Circular dichroic spectra of the polymer are shown in Figure 7. At pH 10.2, the arsanilazotyrosyl chromophores are optically active with a positive ellipticity band at 325 mμ, corresponding to the absorption maximum of the azo chromophore. In addition, a broad, negative ellipticity band, centered at approximately 425 mμ, coincides with the long-wavelength shoulder and tail of the absorption spectrum. The negative, *intrinsic* Cotton effect is consistent with the demonstration of helical regions in the homopolymer (Fasman *et al.*, 1964; Beychok and Fasman, 1964).

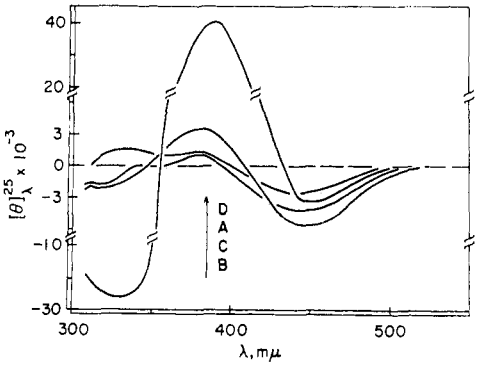

FIGURE 6: Circular dichroism of azoovalbumins in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°). Protein modification, sample preparation and letter code as described for Figure 3.

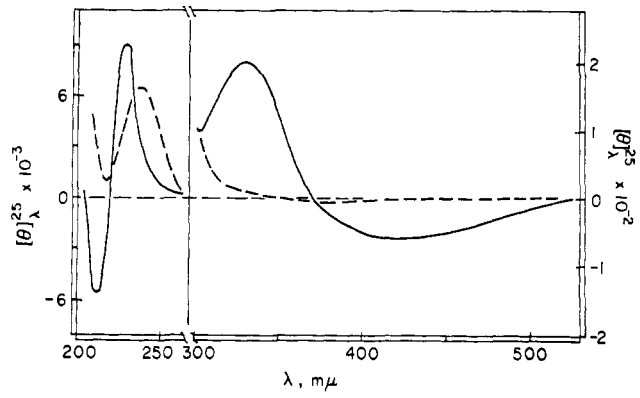

FIGURE 7: Effect of pH variation on circular dichroism of poly-L-tyrosine containing an azotyrosyl chromophore in 0.10 M NaCl, pH 10.2 (———), pH 11.2 (– – – –); [Θ] expressed in units of deg cm² per dmole of tyrosine.

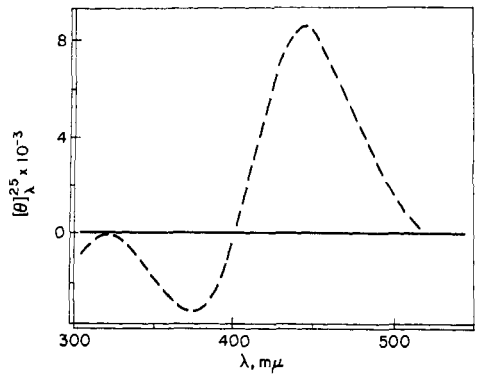

FIGURE 8: Circular dichroism of azoribonuclease in 0.10 M NaCl–0.05 M Tris buffer (pH 7.5, 25°) in the presence (———) and absence (– – – –) of 6 M guanidine hydrochloride. Ribonuclease (2×10^{-4} M) modification was carried out with diazotized *p*-arsanilic acid (2×10^{-3} M) for 30 min in 0.67 M bicarbonate buffer (pH 8.8, 0°); the sample was then separated from the reaction mixture by gel filtration.

Increase in pH can transform poly-L-tyrosine from a helical to a random coil conformation (Fasman *et al.*, 1964). The effect on circular dichroism of the azo polymer by increasing the pH from 10.2 to 11.2 is apparent from Figure 7. The negative, intrinsic Cotton effect disappears, consistent with a change to more random structure; concomitantly, both the positive and negative dichroic bands of the azo chromophore are obliterated.[1] Arsanilazo-*N*-acetyl-L-tyrosinamide displays circular dichroic bands of like sign and position between 300 and 500 mμ but smaller than those of the polymer, and there are no significant alterations on increasing the pH from 7.5 to 12.5. Hence, conformational features would appear to account for the behavior of the ellipticity bands of the azotyrosine polymer.

As might be anticipated, the azo chromophores of proteins appear to be equally sensitive to gross changes in conformation. Thus, at pH 7.5 in 6 M guanidine hydrochloride, the characteristic circular dichroic spectrum of azoribonuclease is obliterated completely (Figure 8), while the corresponding absorption spectrum is not altered significantly. On removing the denaturing agent by dialysis, the distinctive ellipticity bands reappear which are characteristic of native conformation. In 6 M guanidine hydrochloride, the striking differences between the circular dichroic spectra of azo-α-chymotrypsin and azoelastase are destroyed similarly (Figure 2); only a weak positive band remains at 325 mμ, and this is similar in sign and magnitude to that observed for the monoazo derivative of *N*-acetyl-L-tyrosinamide. Therefore, as with the tyrosine polymer, the characteristic, detailed features of the dichroic spectra of the azo proteins appear to depend upon the integrity of three-dimensional, macromolecular structure.

Discussion

Most modifications of proteins with organic reagents depend on the nucleophilic or electrophilic characteristics of the reactants or on their capacity to be oxidized. Hence, based solely on chemical considerations, group specificity and selectivity in native proteins would be expected to be much less frequent and distinctive than is actually observed. Compared to amino acids and peptides both the reactivities of protein side chains and their rates of modification may differ drastically. The detailed bases of these phenomena, quite typical of native proteins, are largely unknown, but must be related to their conformations, since denaturation abolishes these features. Steric factors, location of groups within the three-dimensional structure, their protection by cofactors or substrates, hydrogen bonding, neighboring charges, and the polarity of the environment may all critically affect the reactivity of a group to varying degrees. Similarly, the capacity of the modifying agent to react may be favored or retarded by the same factors, resulting in adsorption, steric hindrance, repulsion, or attraction of the agent. Thus, different protein conformations can influence the ultimate occurrence and rate of modification of a given residue significantly (Cohen, 1968).

Exceptional chemical reactivity has often been observed for residues involved in biological activity, and this correlation can serve to localize a modified residue, albeit tentatively, with respect to the active center of an enzyme. However, in the absence of functional changes, the criteria which might define the basis for selective chemical reactivities, or stereochemical properties, if any, of the modified product are inadequate. This is true particularly when the three-dimensional structure of a protein, and hence, the environmental features affecting the reactivities of side chains, are unknown. Experimental means, presently available to discern reactions and based primarily on conformational features of protein structure, are still very limited in range and scope.

It would appear that diazonium salts can offer new opportunities for additional information bearing on these problems. Azo coupling has been employed extensively in the past to study the composition, structure and immunological properties of proteins, and it is well known that diazonium salts can react readily with histidyl, tyrosyl, lysyl, and perhaps other amino acid side chains. Even though the visible spectra induced on reaction of diazonium salts with proteins could potentially provide a convenient major analytical asset,

[1] Upon further increase to pH 12.5 a small negative band (not shown) appears at 450 mμ.

the poor resolution of the spectral bands of the products has limited the suitability of these salts as site-specific protein reagents.

In efforts to reevaluate the selectivity and specificity of diazonium salts, we have now found that the azo derivatives formed upon modification with the reagents derived from *p*-arsanilic acid, *p*-aminobenzoic acid, 5-amino-1*H*-tetrazole, and 5-aminoindazole can serve as useful optical probes both of chemical modification and of protein conformation. The chromophoric azo derivatives which result from the modification of relatively small numbers of protein groups (Tables I and III) are optically active, generating highly specific *extrinsic* Cotton effect systems in the visible region of the spectrum (Figures 2, 4, and 6 and Table II). Extrinsic Cotton effects in proteins have been observed frequently on formation of reversible protein–chromophore complexes, but the instances in which they accompany *covalent* modifications are still very few (Dowben and Orkin, 1967; Beaven and Gratzer, 1968; Johnson *et al.*, 1968; Meloun *et al.*, 1968; Kagan and Vallee, 1969; Sigman *et al.*, 1969), and systematic studies employing such covalent conformational probes do not seem as yet to have been reported.

Modification with the same diazonium salt, *e.g.*, diazotized *p*-arsanilic acid, of at least a dozen different proteins results in circular dichroic spectra of these azo proteins, each different from one another (Table II). The details of the Cotton effects, their spectral locations, and the signs and magnitudes of the composite bands, appear highly characteristic for each of the particular proteins studied. These differences are most remarkable in view of the frequent similarity of the corresponding absorption spectra (Figures 1, 3, and 5). While the relative orientations of vicinal groups with respect to one another may not be readily discernible in the absorption spectra, they may contribute importantly to the steric factors reflected in optical activity. The variety in three-dimensional, structural disposition of residues within different native proteins could easily condition such varied circular dichroic spectra. Considered in such light, the loss of characteristic features of circular dichroic spectra upon the denaturation of azo proteins (Figure 8) is not unexpected and reflects the dependence of local three-dimensional features upon the maintenance of an intact, overall protein structure.

On the other hand, modification with different diazonium salts of a particular protein can generate apparently closely related azo proteins. When ribonuclease and ovalbumin are coupled with four diazonium salts, differing in both size and charge, the resultant families of absorption spectra are closely similar for all (Figures 3 and 5). However, the circular dichroic spectra of the different azo derivatives of each protein are remarkably similar although distinctive for the two proteins (Figures 4 and 6). The similarity of positions and signs of the dichroic bands within each family of azo proteins suggests an important role for the *protein* in conditioning the details of the ellipticity spectra (Figures 4 and 6). As in all instances, but a small number of residues are modified chemically, the common pattern observed, *e.g.*, in the azo-ribonuclease derivatives, might be expected if the reacting group(s) and their microchemical protein environment were closely similar in all instances.

The spectra of each family of azo proteins differ from one another primarily in band magnitude. In each case, the relative order of the magnitudes of the ellipticity varies from that of the corresponding absorptivity values for the various azo derivatives of a given protein (Figures 3–6). The possible reasons for such variation are many, of course, and the actual basis for these observations can only be conjectured upon, but might include: (1) modification of the same type of residue but in different locations of the protein; (2) modification of the same residue(s) to various degrees; and (3) modification of the same residue to the same degree but resulting in a different signal, conditioned by the features of the interaction with the particular protein environment. Whatever the source of variation, the details would likely be different in each case examined; significantly the circular dichroic spectra provide discrimination not apparent in the absorption spectra.

Several general mechanisms have been proposed to account for the generation of Cotton effects. For strong transitions ($\epsilon > 10^3$), dipole–dipole coupling is considered to play a prominent role in the development of optical activity (Tinoco, 1962; Schellman, 1968). At wavelengths longer than 300 mμ the absorption spectra of azo analogs of tyrosyl, histidyl, and lysyl residues all exhibit absorption maxima with molar absorptivities greater than 10^4 (Tabachnick and Sobotka, 1959; Horinishi *et al.*, 1964; Sokolovsky and Vallee, 1966). Dipole–dipole coupling between the strongly absorbing azo chromophores and the side-chain chromophores of the native amino acids, *e.g.*, tyrosine and tryptophan, represents one mechanism which could generate optical activity in the protein azo chromophores. Similarly, interactions both with the chromophores of the peptide backbone and with other neighboring polarizable groups in the far-ultraviolet spectral region could be considered.

For weak absorption bands, optical activity is considered to arise by either the one-electron mechanism. the μ–m mechanism, or a combination of these (Woody and Tinoco, 1967; Schellman, 1968). It has been shown that the weak transitions of the nonbonding nitrogen electrons in aliphatic azo compounds can be rendered optically active by asymmetry about adjacent carbon atoms (Severn and Kosower, 1969). Similarly, sensitivity of the diazene-bond electrons in the protein azo chromophores to local asymmetry also could contribute to the circular dichroism of these azo chromophores. The possibility of such different modes of interaction between azo chromophores and their protein environment would form a basis for the conformational sensitivity and discrimination observed in the circular dichroic spectra of azo proteins.

The absorption spectra above 300 mμ, both at pH 7.5 and in alkali, suggest the formation of monoazotyrosyl derivatives in most of the proteins here examined. Both the majority of the azo proteins and model monoazotyrosyl derivatives exhibit absorption maxima near 325 mμ, thought characteristic of this species. Therefore, it seems probable that the preponderance of the optically active bands clustered between 320 and 340 mμ (Table II) arise from transitions associated with the monazotyrosyl chromophore in proteins. This view is strengthened by the data obtained with a poly-L-tyrosine polymer, containing azotyrosyl chromophores, where the dichroic maximum at 325 mμ is similar to that seen in many proteins. However, the spectral overlap observed for the common azo derivatives of tyrosine, histidine, and lysine above 350 mμ complicate the assignment of the optically

active bands of the proteins in this spectral region to particular types of modified residues based solely on spectral positions.

Hence, definitive assignments of all wavelength bands to particular residues awaits further characterization of the modified proteins and examination of systems in which unique modification of particular groups can be performed unambiguously. The location of the azoamino acid derivatives in the primary sequence of some of these proteins and the effects of these modifications on enzymatic function constitutes a continuing study in this laboratory. It would appear that such optically active azo protein derivatives should prove valuable in future studies of the overall and local conformations of proteins.

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Myosin Structure as Revealed by Simultaneous Electrophoresis of Heavy and Light Subunits*

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ABSTRACT: A method for the simultaneous resolution of the sulfonyl derivatives of the large and small subunits of myosin on sodium dodecyl sulfate acrylamide gels is presented. DEAE-Sephadex chromatographed myosin (myosin-II) isolated from chicken or rabbit muscle contains only two low molecular weight components when extracted at low pH (6.2) and high ionic strength (μ = 0.5) in the absence of magnesium ions. Myosin-I, extracted by high pH (9.5) and low ionic strength (μ = 0.10), containing magnesium, shows three major and one minor low molecular weight components after chromatography on DEAE-Sephadex. Of the two additional components extracted at high pH, one is tentatively identified as actin. The low molecular weight components released from myosin by sodium dodecyl sulfate treatment are identical with those released by exposure to a pH greater than 10.5. Molecular weight estimates for the two low molecular weight components of myosin-II are made from their electrophoretic mobilities on acrylamide gels calibrated for molecular size. Preliminary molecular weight estimates for the two low molecular weight components are 18,500–19,500 and 32,100–33,000, respectively.

Recent work on the structure of the myosin molecule suggests that the molecule is composed of at least two low molecular weight protein chains (LMP)[1] of average molecular weight 20,000–30,000 (Gershman *et al.*, 1966; Frederiksen and Holtzer, 1968) in addition to the two heavy or fibrous (f) subunits of molecular weight 200,000 (Kielly and Harrington, 1960; Gazith *et al.*, 1970; Frederiksen and Holtzer, 1968). The LMP components are released from the parent molecule by a number of different procedures: treatment with urea or guanidine hydrochloride, exposure to alkaline solutions above pH 10.5, chemical modifications involving

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[1] LMP = low molecular weight protein.