Solution Topography of Proteins by Charge Transfer. Model Complexes, Ribonuclease, and Lysozyme[1]

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Abstract: Fully exposed aromatic amino acid residues on the surface of proteins are potential π donors for a variety of charge-transfer acceptors. Tryptophan and tyrosine and several of their derivatives, as well as histidine and phenylalanine, all form weak intermolecular complexes with the acceptor 1-methyl-3-carbamidopyridinium chloride. The association constants of various tryptophan and tyrosine complexes increase markedly with increasing negative charge on the donor, but only relatively small changes are observed in the corresponding charge-transfer spectra. Tryptophan complexes show no variation in association constants or spectra over a broad pH range, but at high pH the aromatic donor moiety of tyrosine ionizes and the spectral properties of the complex are drastically altered. Histidine and phenylalanine complexes do not absorb appreciably in the spectral region suitable for protein charge-transfer titrations and form very much weaker complexes than do tryptophan or tyrosine. Using the properties of the model complexes as a guide, a detailed spectral analysis has permitted identification of the number and type of surface available donor residues on two proteins. Three of the six tyrosine residues in ribonuclease are accessible to 1-methyl-3-carbamidopyridinium chloride, in excellent agreement with predictions based on the X-ray crystal structure model. In chicken egg-white lysozyme a single tryptophan residue and a single tyrosine residue are available for a charge-transfer interaction in solution. A highly overlapping face-to-face contact between the donor and the acceptor appears to be an essential requirement for efficient charge transfer of the type described here ($\pi_D - \pi_A^*$), and protein donor residues which give rise to a charge-transfer interaction in solution must be either completely exposed or, in a depression, open to solvent of at least the same dimensions as the acceptor (roughly $9 \times 7 \times 4$ Å). The present results demonstrate the feasibility of quantitative determinations of the number and kinds of fully exposed aromatic side chains on proteins in solution.

M ost aromatic amino acids may remain at least partly buried in protein interiors because of their hydrophobic nature, and those which are solvent available are sometimes functionally important. Solvent perturbation and chemical modification techniques are commonly used to determine the availability of aromatic residues in proteins, and a charge-transfer probe (1-methyl-3-carbamidopyridinium chloride; 1 methyl-nicotinamide chloride; MNCl[2]) has recently been used for the same purpose.[3] In contrast to the more traditional techniques, the charge-transfer method appears to be capable of detecting only fully exposed aromatic amino acid side chains. Furthermore, a considerable amount of molecular detail is available concerning the possible geometry of such complexes.[4-6]

In the present study, the effects of the net charge of the donor molecule and of the pH and ionic strength of the medium on the charge-transfer interaction between 1-methylnicotinamide and the aromatic amino acids are described.[7] Tryptophan and tyrosine model donors with covalently linked charges are used to simulate donor amino acids in charged environments on protein

surfaces, and the effects of such charges on the association constant and the absorption spectra of the complexes are considered in detail. There are two reasons for interest in this effect. First, evidence indicates that the dipole orientation and ring overlap are optimized in both inter- and intramolecular indole–MNCl complexes,[4,5,8] and the possible effects of charge differences are therefore of interest in terms of the complex geometries. Second, differences in the charge-transfer parameters which can be correlated with environmental variations in model systems may help in the identification of donor residues on protein surfaces in solution and therefore in the comparison of the solvent availability of such residues in solution and in the crystal. The geometrical requirements for a charge-transfer interaction of the type considered here ($\pi_D - \pi_A^*$) appear to be very specific.[5,6] A full ring face of the donor residue must be available for binding the acceptor, and therefore it is anticipated that some of the more subtle details of surface topography of proteins in solution may be elucidated by this technique.

Since both phenyl and imidazole side chains show an intramolecular charge-transfer interaction with the nicotinamide cation,[9] the possibility of a measurable intermolecular charge-transfer interaction between MNCl and phenylalanine or histidine in solution was investigated.

Intermolecular complexes between MNCl and tyrosine in proteins have not been previously described in the literature. To gain insight into the nature of tyrosine–MNCl complexes in proteins in the absence of interfering groups (tryptophan), the solvent availability of tyrosine residues in ribonuclease, which does not contain tryptophan, was examined by the charge-

(1) This research was supported by NSF Grant GB 18016.

(2) Abbreviations used: MNCl, 1-methyl-3-carbamidopyridinium chloride (1-methylnicotinamide chloride); Trp, tryptophan; Tyr, tyrosine; NAG, N-acetylglucosamine.

(3) (a) D. A. Deranleau, R. A. Bradshaw, and R. Schwyzer, *Proc. Nat. Acad. Sci. U. S.*, **63**, 885 (1969); R. A. Bradshaw and D. A. Deranleau, *Biochemistry*, **9**, 3310 (1970); (b) F. M. Robbins and L. G. Holmes, *J. Biol. Chem.*, **247**, 3062 (1972).

(4) D. A. Deranleau and H. E. Bosshard, manuscript in preparation.

(5) J. R. Herriott, A. Camerman, and D. A. Deranleau, *J. Amer. Chem. Soc.*, **96**, 1585 (1974).

(6) R. Foster, "Organic Charge Transfer Complexes," Academic Press, New York, N.Y., 1969.

(7) D. A. Deranleau and R. Schwyzer, *Biochemistry*, **9**, 126 (1970), have examined several indole–pyridinium complexes in 1% ethanol including two of those presented here. Because of the presence of an organic solvent at significant (molar) concentration, their results may not be appropriate for use in the interpretation of quantitative data obtained with proteins.

(8) S. Shifrin, *Biochim. Biophys. Acta*, **81**, 205 (1964).

(9) S. Shifrin, *Biochemistry*, **3**, 829 (1964).
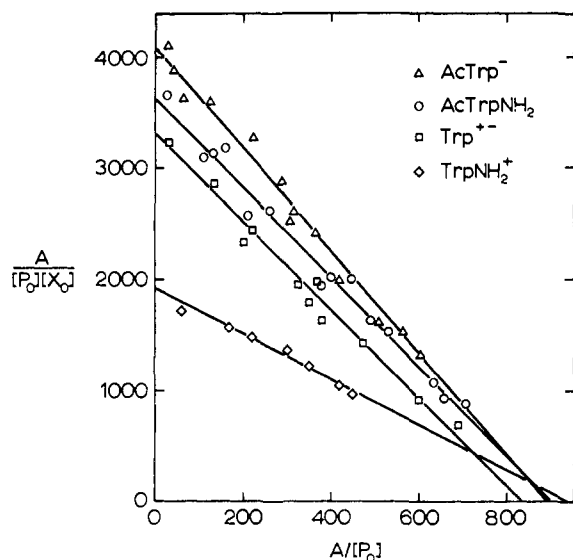
Figure 1. Double intercept plot of constant ionic strength titrations of various tryptophan derivatives with 1-methylnicotinamide chloride (measurement wavelength 350 nm, pH 7.0, 25°, ionic strength 1 M). Donors: N-acetyl-L-tryptophan ($\triangle$), N-acetyl-L-tryptophanamide ($\bigcirc$), L-tryptophan ($\square$), L-tryptophanamide ($\diamond$).

transfer method and the results are interpreted in terms of the X-ray crystal structure model. A careful analysis of the charge-transfer spectrum of lysozyme, which was previously thought to have a single fully available tryptophan residue, revealed the presence of a tyrosine residue as well. The lysozyme results are also interpreted on the basis of X-ray studies, and the possible role of the available tryptophan residue in both lysozyme and α-lactalbumin is discussed.

Experimental Section

Materials. N-Acetyl-L-tryptophan and N-acetyl-L-tryptophanamide were prepared from L-tryptophanamide purchased from Fluka. N-Acetyl-L-tyrosine and N-acetyl-L-tyrosinamide were purchased from Calbiochem and Fox Chemicals, respectively. L-Tyrosinamide, L-histidine monohydrochloride monohydrate, and L-phenylalanine were Mann preparations. 1-Methylnicotinamide chloride was synthesized by the method of Karrer, et al.[10] All buffers were prepared with reagent grade chemicals. Bovine pancreatic ribonuclease A and chicken egg-white lysozyme were both purchased from the Worthington Biochemical Corp. Aqueous solutions of ribonuclease were heated to 60° for 5 min to remove any aggregates, filtered, and adjusted to pH 7.0 just prior to titration with 1-methylnicotinamide. Lysozyme solutions were similarly filtered and brought to pH 7.0 prior to titration.

Methods. The pyridinium-type acceptor 1-methylnicotinamide chloride and an aromatic donor ring form a yellow complex in solution with a long wavelength absorption band separate and distinct from the normal absorption band of either component. This absorption has been attributed to a charge-transfer transition and the properties of the complex can be measured by a titration method. Binding studies in the current work were carried out according to either one or both of two methods. In the *sequential method*[3a] weighed amounts of solid MNCl were added successively to 2 ml of a donor solution and the increase in absorbance was monitored in the spectral range 550-300 nm. A single wavelength, usually 350 nm, was chosen for initial analysis of the titration data. The association constant k and the extinction coefficient ϵ for the complex at the wavelength chosen were calculated from the slope and intercepts of a plot of $A/[P_0][X]$ vs. $A/[P_0]$ according to the relationship

$$A/[P_0][X] = k[\epsilon - (A/[P_0])] \qquad [X] \simeq [X_0]$$

(10) P. Karrer, G. Schwarzenbach, F. Benz, and U. Solmssen, *Helv. Chim. Acta*, **19**, 811 (1936).

where [P_0] and [X_0] are the total donor and acceptor concentrations, respectively, and A is the absorbance corrected for excess acceptor and for volume changes.

When binding of the probe is affected by changes in the ionic strength of the solution, anomalous values for the association constant and extinction coefficient of the complex result if sequential titration data are analyzed by linear fitting techniques. In such cases a *nonsequential method* was employed in which the ionic strength was maintained at a constant level by the addition of KCl. Stock solutions of 2 M KCl and 2 M MNCl were prepared, and aliquots of each were added in varying proportions to 1.0 ml of a donor solution of known concentration in a 2.0-ml volumetric flask. The aliquots of the KCl and MNCl solutions were chosen in such a fashion so as to not exceed a 1-ml total, and so that, on making the final solution up to volume, a constant ionic strength of 1 M was achieved in all cases. In practice, less than 10 μl of solvent was necessary to bring the volume up to 2.0 ml, so the volumes in this particular case are approximately additive. The absorbance of these solutions was recorded as a function of wavelength, and the data were analyzed in terms of a plot of $A/[P_0][X]$ vs. $A/[P_0]$ as described above.

The solutions of model donor compounds were prepared by weight and ranged from 10^{-4} to 10^{-3} M for tryptophanyl derivatives, and from 10^{-3} to 5×10^{-2} M for other model donors. In each titration the MNCl concentration was varied from 0.05 to 1 M. For ribonuclease-MNCl and lysozyme-MNCl binding studies, protein concentrations were determined from the published extinction coefficients $\epsilon(278) = 10,000$ cm^2/mmol for ribonuclease and $\epsilon(282) = 37,900$ cm^2/mmol for lysozyme. All spectra were recorded on a Cary Model 15 spectrophotometer in which the sample and reference solutions were maintained at 25° by means of thermostated cell holders.

The pH of the titrations was controlled by careful pH adjustment of unbuffered solutions or by the use of 0.1 M buffers. Both methods proved to be equivalent, and different buffers at the same pH did not give rise to noticeable changes in the results. When 0.1 M buffers were used in the nonsequential method, the ionic strength was increased over 1 M by the ionic strength of the buffer. No detectable changes in the results could be observed as a consequence of this small ionic strength increase at high total ionic strength.

The pK_a of the phenolic group of N-acetyl-L-tyrosinamide was spectroscopically determined using the method of Wetlaufer.[11] Concentrations of the two tyrosine species were compared using $\epsilon_{Tyr}(275) = 1340$, $\epsilon_{Tyr^-}(290) = 2300$, and the isosbestic $\epsilon_i(277) = 1290$ cm^2/mmol. Experiments in 0, 0.1, and 1.0 M KCl gave pK_a values of 10.1, 10.0, and 9.9, respectively.

The stability of MNCl in base was investigated spectroscopically using several concentrations of MNCl between 0.05 and 1.0 M. The pH of these solutions was adjusted from neutrality up to its final value, and the absorption spectrum was measured immediately. The solutions were then allowed to stand for at least 2 hr and then the spectrum was remeasured.

Difference spectra for each donor-acceptor system were measured from 550 to 290 nm in double tandem cells. The difference spectrum for the N-acetyltyrosinamide anion-MNCl complex was deduced from a pH 10.8 difference spectrum. The spectra obtained in this fashion were converted into extinction spectra by normalization to single wavelength extinction values determined from binding studies.

Results and Discussion

Effects of Local Donor Environment. The charge-transfer interactions of MNCl with several tryptophan and tyrosine donors—selected to mimic solvent available residues on proteins—were studied at neutral pH. If a particular residue in a protein has only uncharged neighboring residues, its environment can be simulated by an appropriate N-acetylamino acid amide, and the same residue with neighboring positive or negative charges can be approximated (at neutral pH) by respectively an amino acid amide or an N-acetylamino acid. For a donor residue in the vicinity of a salt bridge, the zwitterion may be a reasonable model. All four models—the N-acetyl, amide, and N-acetyl amide derivatives and zwitterions of tryptophan and tyrosine—

(11) D. B. Wetlaufer, *Advan. Protein Chem.*, **17**, 303 (1962).

Table I. Spectroscopic Parameters for Model Complexes with N^1-Methylnicotinamide Chloride at pH 7.0

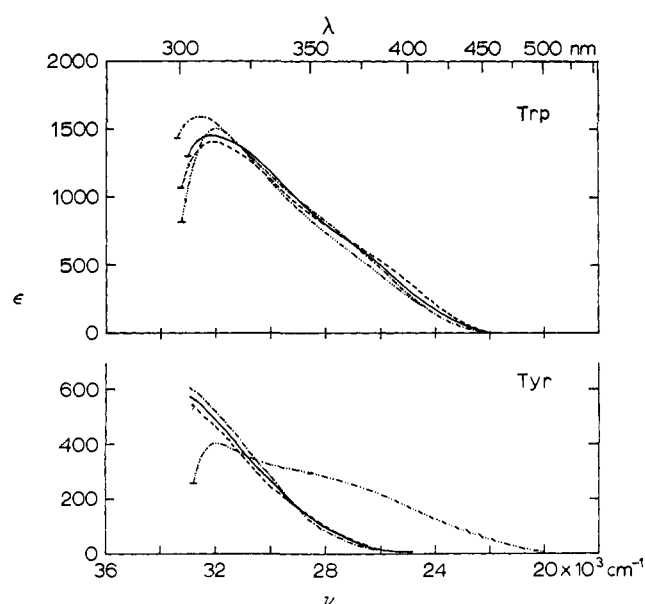| Donor	Net charge	Association constant k (cm³/mmol)	Extinction coeff (cm²/mmol at 350 nm)	Integral area:[a] $10^{-3}\int_\nu \epsilon\ d\nu$
N-Acetyl-L-tryptophan	−1	4.68 ± 0.20	877 ± 37	8.35
N-Acetyl-L-tryptophanamide	0	4.01 ± 0.17	902 ± 43	8.31
L-Tryptophan	0	3.94 ± 0.15	840 ± 36	7.81
L-Tryptophanamide	+1	2.11 ± 0.09	917 ± 41	8.37
	Av values	(3.68)	884 ± 39	8.21 ± 0.20
N-Acetyl-L-tyrosine	−1	1.17 ± 0.08	138 ± 10	1.76
N-Acetyl-L-tyrosinamide	0	0.95 ± 0.07	137 ± 10	1.70
L-Tyrosine	0	0.81 ± 0.06	130 ± 11	1.84
L-Tyrosinamide	+1	0.55 ± 0.08	125 ± 19	1.89
	Av values	(0.87)	132 ± 13	180 ± 0.05
N-Acetyl-L-tyrosinamide anion[b]	−1	1.66 ± 0.18	297 ± 15	2.74
L-Histidine	0	<0.1	50	1.8
L-Phenylalanine	0	<0.1	10	0.8

[a] Integration limits: 20,000–33,000 cm⁻¹ (400–303 nm). [b] At pH 10.8, corrected for contribution from the un-ionized species.
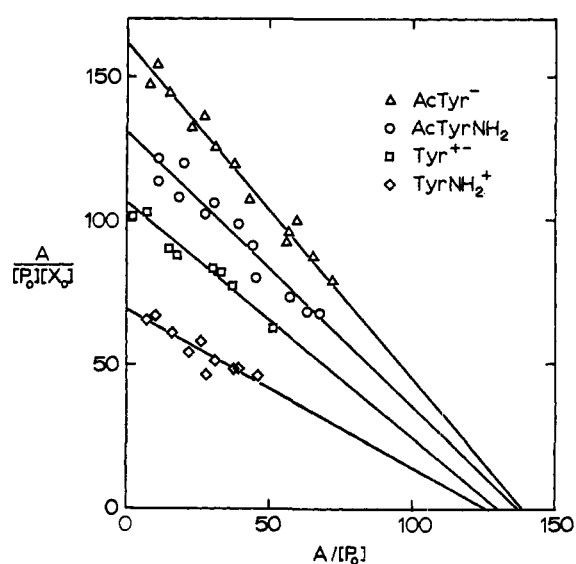
Figure 2. Double intercept plots of constant ionic strength titrations of various tyrosine derivatives with 1-methylnicotinamide chloride (measurement wavelength 350 nm, pH 7.0, 25°, ionic strength 1 M). Donors: N-acetyl-L-tyrosine (△), N-acetyl-L-tyrosinamide (○), L-tyrosine (□), and L-tyrosinamide (◇).

Figure 3. Measurable portion of the charge-transfer bands of 1-methylnicotinamide complexes with various donors: upper curves, tryptophan derivatives; lower curves, tyrosine derivatives. In each case the dashed line is the negatively charged N-acetyl derivative, the solid line is the N-acetylamino acid amide (no charge), and the line with alternating single dots and dashes is the positively charged amino acid amide. The tryptophan zwitterion is represented by (– · · · · –· · · ·), and to avoid confusion the tyrosine zwitterion is not shown. The tyrosine curve (– · · –· ·) extending out to 20,000 cm⁻¹ is the spectrum of the N-acetyl-L-tyrosine double anion (carboxyl and phenolic hydroxyl groups both ionized).

were used to investigate the effects of local donor environment on the properties of the charge-transfer complexes with MNCl.

Double intercept plots for the binding of MNCl to each of the four tryptophan and tyrosine derivatives are shown at neutral pH and constant ionic strength in Figures 1 and 2. The association constant k (negative slope of the plot) clearly increases with the negativity of the donor. Although the figures show double intercept plots at only a single wavelength, it has been verified for all eight derivatives that the association constant is independent of wavelength. Multiwavelength double-intercept plots were prepared at 10-nm intervals from 320 to 450 nm and analyzed by linear least-squares fitting procedures. Within experimental error, the fitted slopes were found to be constant over the entire measured spectral range for any of the model complexes studied. Numerical values of the association constants are listed in Table I. Except for the negatively charged N-acetyl derivatives all model complexes exhibited a

variation in the slope of the plot with increasing ionic strength when the sequential titration was used, and thus the present results—obtained with the nonsequential method—are not necessarily comparable with previously reported values.[7]

The high saturation intercept on the $A/[P_0]$ axis of the double intercept plot is the extinction coefficient ϵ of the charge-transfer complex at a given wavelength. Values of ϵ obtained from multiwavelength plots, along with those determined from normalized difference spectra, provide a more complete picture of the charge-transfer absorption band for model systems. The charge-transfer spectra of the tryptophan and tyrosine model complexes are compared in Figure 3. The

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integrated areas under the absorption bands are listed in Table I for the portion of the spectra which can be measured without serious error ($\lambda > 305$ nm). Possible variations of the band properties with ionic strength were investigated by measuring difference spectra in 0, 0.5, and 1 M KCl. No significant variations in intensity or shifts in spectral position were noted in any case.

From inspection of the figures one can generalize as to the effect of donor environment on the charge-transition at neutral pH. The association constant for both tryptophan– and tyrosine–MNCl complexes increases with increasing electronegativity of the donor, which is reasonable considering that the acceptor is positively charged and that a significant electrostatic contribution to the binding energy should be expected. Within a given donor class, only minor differences occur in the shapes and intensities of the charge-transfer bands. If the observed charge-transfer spectrum is assumed to be due to the sum of contributions from two overlapping Gaussian bands,[7] the differences within a given class appear to relate to the energy separation and relative intensities of the two bands. In the tryptophan series the energy separation is greatest for the uncharged derivative (N-acetyltryptophanamide) and least for the zwitterion (tryptophan). The long wavelength band appears to shift to slightly higher wavelengths with increasing negative charge, and the short wavelength band undergoes a concomitant decrease in intensity. While these slight shifts in band position and intensity may give an indication of the polarity of the environment of an exposed tryptophan or tyrosine residue in a protein, their magnitudes are too small to be used with certainty in such an assignment. On the other hand, the similarity in the spectra (with the possible exception of the zwitterion) suggests that any of the models, or an average spectrum of all of them, may be used in obtaining estimates of the number of tryptophan and tyrosine residues contributing to the charge-transfer spectrum of a particular protein. A final choice of models suitable for the fitting of protein data can be made more easily on the basis of the association constants, which are clearly more sensitive to variations in local charge environment.

The relative constancy of the integrated area under the absorption band in the two classes of models—despite changes in the local donor environment and in the association constants—indicates a high specificity for the orbital interaction between the donor and the acceptor rings. This observation may be interpreted as support for the hypothesis[4,5] that these complexes are characterized by fixed and relatively unique donor–acceptor geometries, in which in-plane rotational isomerism is severely limited by the mutual interaction of the permanent dipoles of the donor and acceptor rings.

Histidine and Phenylalanine Complexes. Intramolecular complexes incorporating the imidazole and phenyl rings as donors with the carbamidopyridinium acceptor exhibit charge-transfer bands similar to those observed with tryptophan and tyrosine donors.[9] To investigate the possibility of observing intermolecular complexes between protein histidyl and phenylalanyl residues and MNCl, charge-transfer titrations were carried out using L-histidine and L-phenylalanine as donors. In both cases, evidence for complex formation was obtained from difference spectra but the binding

was so weak that accurate measurement of either the association constants or the extinction coefficients was not possible. The association constants were estimated to be less than 0.1 cm^3/mmol in both cases. This means that at the highest concentrations of MNCl available (~ 1 M), not more than about 10% saturation of the donor can be obtained, compared to roughly 50% for tyrosine donors and 80% for tryptophan donors. Furthermore, neither histidine nor phenylalanine complexes absorb appreciably above 350 nm ($\epsilon < 50$ at 350 nm in both cases, or about one-third of the extinction of tyrosine complexes and less than 6% of the extinction of tryptophan complexes at this wavelength). Taken together, the very small association constants and the spectral distribution of the absorbance of histidine and phenylalanine complexes ensure that at wavelengths above about 330 nm, contributions to the protein charge transfer band from these two residues will normally be negligible. Table II illustrates the relative sensitivity

Table II. Relative Sensitivity for the Detection of Various Model Complexes at Specified Wavelengths

Donor	Relative sensitivity (ratio of $k\epsilon$) at wavelength indicated			Max obtainable saturation fraction,[a] $\hat{s}$	% of information obtainable[b]
	330 nm	350 nm	380 nm		
N-Acetyl-L-tryptophanamide	1.0	1.0	1.0	0.8	89
N-Acetyl-L-tyrosinamide	0.06	0.04	0.008	0.5	50
L-Histidine	0.005	0.001	0.0002	0.09	3
L-Phenylalanine	0.0008	0.0005		0.09	3

[a] The saturation fraction obtainable at 1 M MNCl. [b] $100 \hat{I}/I_{max}$, where $\hat{I}$ is the information (in bits) obtainable at $\hat{s}$, and I_{max} is the total information contained in the binding curve (when s varies continuously from 0 to 1); see ref 17 for details.

of tyrosine, histidine, and phenylalanine complexes in comparison to the tryptophan complexes, using the product $k\epsilon$ as a measure of sensitivity.

pH Effects. The chemical instability of pyridinium salts in base provides a practical upper limit for the pH range of charge-transfer titrations using MNCl as an acceptor. Several investigators have studied the interactions of pyridinium salts with the hydroxyl ion,[12] and a 290-nm absorption band with a long tail into the visible region of the spectrum has been observed in weak base. This band appears quickly and has been attributed to a charge-transfer transition between OH^- and the pyridinium ring. With time and under more severe conditions a new absorption band appears at 360 nm which is related to an irreversible structural change. Proposed mechanisms for and the kinetics of these reactions are described elsewhere.[12] For our purposes, it is important to ascertain the pH at which the absorbance due to such side reactions begins to interfere with the measured spectra of the charge-transfer complexes under investigation. No MNCl spectral changes were noted at 25° in 2 hr, the time period required for an average titration experiment, at pH values below 11. Above pH 11, however, spectral data show that MNCl decom-

(12) R. M. Burton and N. O. Kaplan, *Arch. Biochem. Biophys.*, **101**, 139 (1963); R. B. Martin and J. G. Hull, *J. Biol. Chem.*, **239**, 1237 (1964).

position proceeds rapidly, and therefore no pH higher than 10.8 was used in the present work.

The uncharged derivatives, N-acetyl-L-tryptophan-amide and N-acetyl-L-tyrosinamide, were chosen to investigate the possible effects of pH on protein charge-transfer complexes. Between pH 2 and 10, no significant changes in association constant or extinction coefficient at 350 nm were observed for the tryptophan derivative, and, similarly, no changes were observed between pH 2 and 8 for the tyrosine derivative. The remarkable constancy in the absorption intensity of these charge-transfer complexes at varying pH values again suggests that the complex geometry remains essentially unaltered under a wide variety of conditions.

Above about pH 8, the phenolic hydroxyl of tyrosine begins to ionize, and the aromatic donor moiety at high pH is the phenolate ion. A phenolate anion–MNCl charge-transfer spectrum was previously noted by Shifrin in an intramolecular donor–acceptor complex.[9] The extinction coefficients and association constants for the ionized intermolecular complex can be deduced from charge-transfer titrations at constant pH and ionic strength as follows. Let [HPX] stand for the concentration of the complex between the protonated donor HP and the acceptor X and [P⁻X] for the concentration of the unprotonated donor P⁻ with the acceptor. Defining the pertinent association constants by the relations

$$[HPX] = k[HP][X]$$

$$[P^-X] = k'[P^-][X]$$

$$[HP] = k_a[H^+][P^-]$$

$$[HPX] = k_a'[H^+][P^-X] \tag{1}$$

it can be shown that the absorbance per mole of total donor is given by

$$\frac{A}{[P_0]} = \frac{(\epsilon k k_a[H^+] + \epsilon' k')[X]}{1 + k'[X] + k_a[H^+](1 + k[X])} \tag{2}$$

where primed values refer to the ionized form of the donor as in eq 1. Furthermore, at constant pH the (negative) slope of a plot of $A/[P_0][X]$ vs. $A/[P_0]$ is linear and equal to

$$(k' + kk_a[H^+])/(1 + k_a[H^+]) \tag{3}$$

and the $A/[P_0]$ axis intercept of the plot is, from eq 2[13]

$$\lim_{[X]\to\infty} \frac{A}{[P_0]} = \frac{\epsilon'k' + \epsilon k k_a[H^+]}{k' + kk_a[H^+]} \tag{4}$$

The association constant and extinction coefficient derived from a constant pH double-intercept plot at 350 nm are shown in Table I for the N-acetyl-L-tyrosinamide anion–MNCl complex, and the spectrum of the ionized complex is shown in Figure 3.

Protein Studies. The analysis and interpretation of charge-transfer data from proteins are inherently more complicated than for model complexes, since several

binding sites, each with its own characteristic association constant and extinction coefficient, may be present on the protein surface. For a protein with n such sites, the number of (macroscopic) constants required to fit the charge-transfer data to an exact binding equation is 2n. Generally speaking, the accuracy of the data obtained with proteins does not appear to warrant fitting more than four constants. Thus, for a protein with three or more binding sites, it is advantageous to make use of approximate methods of analysis which require fewer constants in the fitting procedure. Without going into details, which will be given elsewhere,[14] one such approximate method seems to be adequate for use in protein charge-transfer systems. By assuming that the sites are *independent* of one another, they will be either *all* identical or will fall into several *classes* of identical sites, and the charge-transfer titrations will be described by the *observation equations*[14]

$$A/[P_0] = n\epsilon k[X]/(1 + k[X]) \quad \text{(identical sites of a single class)} \tag{5}$$

$$A/[P_0] = \sum_i n_i\epsilon_i k_i[X]/(1 + k_i[X]) \quad \text{(identical sites of several classes } i) \tag{6}$$

where n is the number of sites in a given class, e.g., tryptophan residues, and k and ε are the *apparent microscopic* (site-specific) association constants and extinction coefficients. The approximate treatment has the further advantage that—unlike the treatment in terms of macroscopic parameters—the estimated constants are directly comparable with the constants deduced from 1:1 model complexes (identical macroscopic and microscopic constants, see ref 17).

Ribonuclease. Identical and Independent Sites of a Single Class. Physical and chemical evidence previously obtained with ribonuclease indicates that three tyrosine residues are "solvent-available,"[15] and models based on X-ray crystal structure data[16] are consistent with this finding. Since ribonuclease does not contain tryptophan, and since contributions to the charge-transfer spectrum from histidine and phenylalanine complexes can be ignored under the conditions of measurement (*vide supra*), the charge-transfer band of this protein in the presence of MNCl would be due entirely to exposed tyrosine residues. The charge-transfer titration of ribonuclease with MNCl at pH 7 is shown in Figure 4, and the association constant and spectral properties deduced from the application of the identical and independent site approximation are listed in Table II. The double intercept plot shows no evidence of curvature and the slope does not vary with wavelength, both factors indicating that the equivalent and independent site model is a reasonable approximation in this case.[17]

The magnitude of the extinction coefficient suggests that three tyrosine residues are available for complexation with the probe (see Figure 4). This supposition is confirmed by the spectral distribution of the ribonuclease

(13) When pH = pK_a, $K_a[H^+]$ = 1 and eq 3 and 4 become, respectively

$$\text{slope} = (k + k')/2 \tag{3'}$$

$$\text{intercept} = (\epsilon'k' + \epsilon k)/(k' + k) \tag{4'}$$

The first and last of eq 1 are not independent, and since $kk' = k_a k_a'$, it is possible to deduce both the association constant for the complex of the acceptor with the unprotonated donor (k') and the proton association constant of the charge-transfer complex (k_a').

(14) D. A. Deranleau, manuscript in preparation.
(15) For a recent review, see F. M. Richards and H. W. Wyckoff, "The Enzymes," 3rd ed, Vol. IV, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, p 647, and references cited therein.
(16) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, 242, 3984 (1967); H. W. Wyckoff, D. Tsernoglou, A. W. Hanson, J. R. Knox, B. Lee, and F. M. Richards, *ibid.*, 245, 305 (1970).
(17) D. A. Deranleau, *J. Amer. Chem. Soc.*, 91, 4044, 4050 (1969).
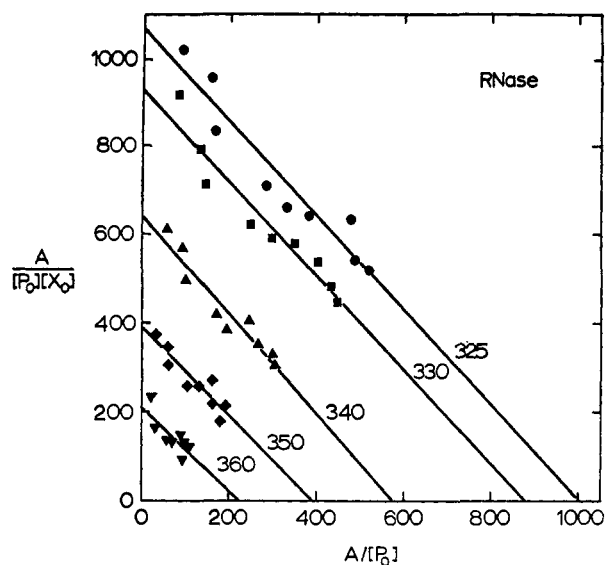
Figure 4. Multiwavelength double intercept plot of the titration of ribonuclease with 1-methylnicotinamide chloride at pH 7.0, 25° (ionic strength 1 M). Measurement wavelengths (in nanometers) are indicated on the plot.

Figure 5. Charge-transfer spectrum of the ribonuclease-MNCl complex (solid line) and the addition spectrum of three N-acetyl-L-tyrosine-MNCl complexes (dashed line). Filled circles with error bars are extinction coefficients calculated from the multiwavelength plot (Figure 4), and the lines are from difference spectra normalized to the extinction coefficient at 350 nm.

charge-transfer band (Figure 5) and the integral area under the band (Table III). The agreement between the

Table III. Spectroscopic Parameters for Protein-MNCl Complexes

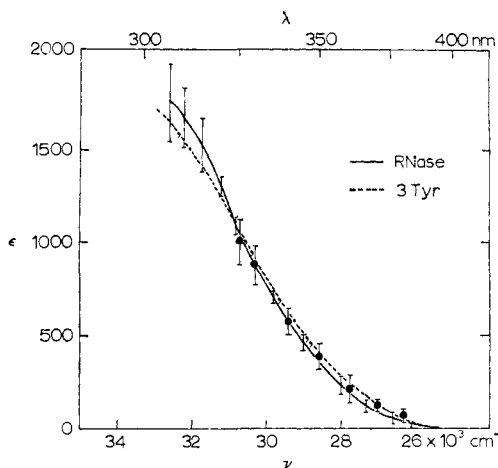| Donor	Av slope of double intercept plot[a]	Extinction coeff (cm²/mmol at 350 nm)	Integral area:[b] $10^{-6}f_\nu\epsilon$ dν
Ribonuclease	1.06 ± 0.14	387 ± 70	3.71
3 × N-acetyl-L-tyrosine	1.17 ± 0.08	414 ± 30	3.64
3 × N-acetyl-L-tyrosinamide	0.95 ± 0.07	411 ± 30	3.74
3 × Tyr (av)[c]	(0.87)	396 ± 39	3.80
Lysozyme	3.19 ± 0.19	1040 ± 25	8.09
N-Acetyl-L-tryptophan + N-acetyl-L-tyrosine	4.32	1015 ± 46	7.93
N-Acetyl-L-tryptophanamide + N-acetyl-L-tyrosinamide	3.60	1039 ± 52	8.14
Trp + Tyr (av)[c]	3.31	1016 ± 52	8.04

[a] Straight lines for ribonuclease and tyrosine models, curves for lysozyme and lysozyme models. The average slope is the average tangent to the curve. [b] Integration limits: 20,000-32,000 cm⁻¹ (note difference from Table I limits). [c] See Table I.

ribonuclease spectrum and the addition spectrum of three tyrosine residues appears to be quite reasonable. The charge-transfer results thus support previous physical and chemical evidence concerning the availability of tyrosine residues in this protein.

It is essential in the formation of charge-transfer complexes of this type that one ring face of the tyrosine residue be more or less completely exposed on the surface of the protein. According to the skeletal model deduced from X-ray crystal structure studies,[16] this would appear to be clearly the case for Tyr 76 and Tyr 115. The third fully exposed residue may be Tyr 92, which is shown with one ring face open to the solvent in both the skeletal[16] and in the space-filling models.[18]

Three tyrosine residues are selectively iodinated in solution at pH 6.7 (Tyr 73, 76, and 115),[19] and two of these (73 and 115) can be iodinated in the crystal as well.[16] Tyr 73 presumably would not form a charge-transfer complex with MNCl because it is oriented roughly perpendicular to the surface of the molecule, and both ring faces are at least partially blocked by overlapping sections of the main chain. On the other hand, iodination of this residue may be sterically possible (aside from the fact that it is iodinated in the crystal) because the hydroxyl group and one edge of the ring protrude into the surrounding solvent. Tyr 92 is also iodinated under slightly different conditions,[19] but since the back surface of the ring is effectively blocked, this substitution would appear to require some movement of the ring, out from Pro 93, in order to provide room for the leaving group. In the presumed charge-transfer complex of Tyr 92 with MNCl, such side-chain movement is probably not necessary.

The picture developed above suggests that the solution conformation of the surface tyrosine residues of ribonuclease closely resembles the conformations of these residues in the crystal. However, our interpretation of the iodination studies implies a certain degree of flexibility in the conformation of some surface residues, depending on external conditions, and emphasizes a chemical reason why a residue may be found to be "solvent-available" by one technique but not by another. Flexibility in the surface residues of proteins has been clearly shown by Tulinsky, et al., in α-chymotrypsin crystals.[20]

Lysozyme. Identical and Independent Sites of More Than One Class. In the original investigation of the interaction of MNCl with chicken egg-white lysozyme,[3a] a tyrosine contribution to the charge-transfer spectrum was overlooked. This can be seen in the fitted spectrum shown in Figure 6 but is not immediately apparent

(18) R. E. Dickerson and I. Geis, "The Structure and Action of Proteins," Harper and Row, New York, N. Y., 1969, pp 79-81.

(19) R. W. Woody, M. E. Friedman, and H. A. Scheraga, Biochemistry, 5, 2034 (1966).
(20) A. Tulinsky, R. L. Vandlen, C. N. Morimoto, N. V. Maki, and L. H. Wright, Biochemistry, 12, 4185 (1973); R. L. Vandlen and A. Tulinsky, ibid., 12, 4193 (1973).

in the double intercept plot at 350 nm.[3a] At this wavelength, the product $n_i \epsilon_i k_i$ is much smaller for tyrosine than for tryptophan complexes, and theoretical double intercept plots based on eq 5 or 6 will be similar up to about half-saturation of the tryptophan residue. Significant curvature would appear only at very high concentrations of MNCl (near the high saturation intercept), and data points are not available in this region. The original interpretation[3a] of the lysozyme titration was based on the double intercept plot derived from eq 5 ($n = 1$ Trp), whereas in actual fact the data are best accounted for using the two-site model implicit in eq 6 ($n_{Trp} = n_{Tyr} = 1$). In retrospect, some curvature can be seen near the high concentration end of the available lysozyme data,[3a] but the significance of this curvature was overlooked by the authors.

Examination of the lysozyme crystal model of Blake, et al.,[21] reveals that in addition to Trp 62, there is a surface tyrosine residue (Tyr 23) which forms an intermolecular contact with Arg 114 of an adjacent lysozyme molecule. Presumably this tyrosine residue would also be available to bind MNCl in solutions of lysozyme. If this is the case, then the second, weak binding site found on *oxidized* lysozyme (Trp 62 destroyed) is probably Tyr 23, rather than Trp 63 as originally suggested.[3a]

The magnitude of the extinction coefficients found by Robbins and Holmes in MNCl titrations of the homologous protein α-lactalbumin[3b] indicates that both a tryptophan and a tyrosine residue are surface available in this protein as well, although these authors also interpreted their data in terms of a single tryptophan residue. If a single tryptophan and a single tyrosine residue are fully exposed on the surface of both lysozyme and α-lactalbumin, then this could be taken as further evidence for the three-dimensional structural homology suggested by Hill, et al.[22]

Chicken egg-white lysozyme and human α-lactalbumin both bind N-acetylglucosamine (NAG), and it is known that the ring nitrogen of Trp 62 in lysozyme is hydrogen bonded to the bound NAG molecule.[3a] Although Trp 62 of lysozyme is replaced by Ile 59 in the homologous position in α-lactalbumin, Trp 63 of lyso-

(21) C. C. F. Blake, G. A. Mair, A. C. T. North, D. C. Phillips, and V. Sarma, *Proc. Roy. Soc., Ser. B*, **167**, 365 (1967); C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. Sarma, *ibid.*, **167**, 378 (1967).
(22) R. L. Hill, K. Brew, T. C. Vanaman, I. P. Trayer, and P. Mattock, *Brookhaven Symp. Biol.*, **21**, 139 (1968).
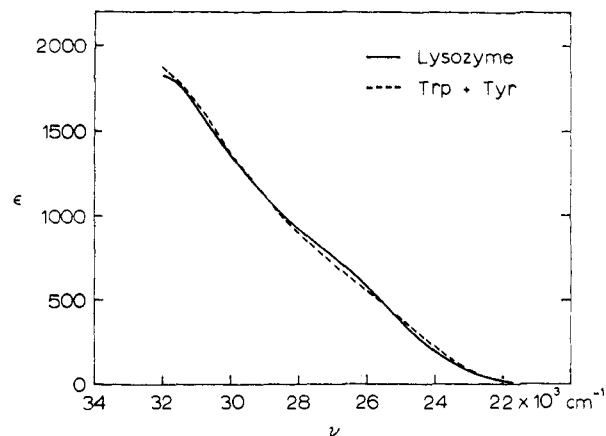
Figure 6. Charge-transfer spectrum of the lysozyme-MNCl complex (solid line, ref 3a) and the addition spectrum of N-acetyl-L-tryptophan plus N-acetyl-L-tyrosine-MNCl complexes (dashed line) at neutral pH, 25°.

zyme is repeated in the homologous position as Trp 60 in α-lactalbumin. It is quite possible that Trp 60 in α-lactalbumin plays the same role as Trp 62 in lysozyme with respect to the binding of NAG—acting as a specific hydrogen bond donor toward the bound NAG molecule.

While we prefer the line of reasoning which suggests that the perturbation of the solution structure is mainly the consequence of the free energy of interaction between the surface residue of interest and the perturbant (and thus that MNCl, with an effectively zero free energy of interaction, is an extremely weak perturbant), our arguments concerning the specific residues involved in the MNCl binding are circumstantial rather than evidentiary. In the absence of explicit chemical evidence identifying the donor residues which constitute the binding sites, one cannot rule out the possibility that even a very weak interaction may still be sufficient to trigger a local conformational change which results in complete exposure of a previously only partially exposed donor residue and that the flexibility of the surface residues of proteins may be far greater than previously suspected.