A Competitive Labeling Method for the Determination of the Chemical Properties of Solitary Functional Groups in Proteins†

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ABSTRACT: The properties of the functional groups in a protein can be used as built-in probes of the structure of the protein. We have developed a general procedure whereby the ionization constant and chemical reactivity of solitary functional groups in proteins may be determined. The method may be applied to the side chain of histidine, tyrosine, lysine, and cysteine, as well as to the amino terminus of the protein. The method, which is an extension of the competitive labeling technique using [^{3}H]- and [^{14}C]1-fluoro-2,4-dinitrobenzene (N_2ph-F) in a double-labeling procedure, is rapid and sensitive. Advantage is taken of the fact that after acid hydrolysis of a dinitrophenylated protein, a derivative is obtained which must be derived from a unique position in the protein. The method has been applied to the solitary histidine residue of lysozyme, α-lytic protease, and *Streptomyces griseus* (S.G.) trypsin, as well as to the amino terminus of the latter protein. The following parameters were obtained for reaction with N_2ph-F at 20°C in 0.1 *N* KCl: the histidine of hen egg-white lysozyme, pK_a of 6.4 and second-order velocity constant of 0.188 M^{-1} min^{-1}; the histidine of α-lytic protease, pK_a of 6.5 and second-order velocity constant of 0.0235 M^{-1} min^{-1}; the histidine of S.G. trypsin, pK_a of 6.5 and second-order velocity constant of 0.0328 M^{-1} min^{-1}; the valyl amino terminus of S.G. trypsin, pK_a of 8.1 and second-order velocity constant of 0.403 M^{-1} min^{-1}. In addition, the results obtained provide clues as to the microenvironments of these functional groups, and indicate that the proteins studied undergo pH-dependent conformational changes which affect the microenvironment, and hence the chemical reactivity of these groups.

The elucidation of the chemical properties of individual functional groups in proteins, and the relationship of these properties to the macrostructure, is fundamental to the understanding of protein structure and function. These properties are determined by the inherent properties of the group concerned, and its interactions with the microenvironment. Ion-pair formation, hydrogen bonding, and hydrophobic interactions should all affect the chemical reactivity of individual functional groups. Thus, from a study of reactivity, we may deduce the local environment of the group. In the case of functional groups which are intimately involved in the bond-making and bond-breaking process, the efficiency of catalysis will be determined by the chemical properties of these groups. Currently, the only method which can be used to directly determine the chemical properties of individual functional groups in proteins is the competitive labeling approach (Kaplan et al., 1971). In principle, it can be applied to any functional group in any protein, and unlike most chemical methods, the structure of the protein is not perturbed by the procedure.

At present, each particular case to be studied represents a unique problem. In this article, we present a development of the competitive labeling method which is experimentally facile, and can, in principle, be applied to a class of problems, viz. the determination of the properties of any functional group which forms an acid-stable derivative with 1-fluoro-2,4-dinitrobenzene (N_2ph-F),[1] and is the sole representative of its class of functional group within the protein. That is, the thiol, imidazolyl, hydroxyphenyl, or ϵ-amino functions of proteins containing a solitary cysteine, histidine, tyrosine, or lysine residue, respectively, or the α-amino group of any protein possessing a unique and unblocked amino terminus.

We have been interested for some time in the properties of the active-site histidine residue of the serine proteases (Cruickshank and Kaplan, 1972, 1974, 1975). Two serine proteases, *Streptomyces griseus* (S.G.) trypsin[1] (Olafson et al., 1975) and α-lytic protease (Olson et al., 1970), each contain a single histidine residue, and these offered good candidates for the application of the new methodology. Furthermore, the histidine residue of α-lytic protease has been studied by ^{13}C nuclear magnetic resonance (NMR) (Hunkapiller et al., 1973), which provided an opportunity to correlate the results obtained by the two techniques.

The properties of catalytic histidines, while being of considerable interest, must be interpreted in comparison with noncatalytic histidines. While studies on histidine-40 of α-chymotrypsin (Cruickshank and Kaplan, 1972) could be considered as being useful in this context, the proximity of this residue to the active site (Sigler et al., 1968) could complicate interpretation. The solitary histidine residue of hen egg-white lysozyme is located on the opposite side of the protein from the active-site cleft (Imoto et al., 1972) and is replaced by a leucine residue in several avian species

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[1] Abbreviations used are: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; S.G. trypsin, *Streptomyces griseus* trypsin; N_2ph, 2,4-dinitrophenyl; Dns-OH, 8-dimethylamino-1-naphthalenesulfonic acid; Dns-Arg, 8-dimethylamino-1-naphthalenearginine.

(Dayhoff, 1972). Thus, it may serve as a good model of a "normal" histidine. In addition, NMR studies on this residue have been carried out (Bradbury and Wilairat, 1967; Meadows et al., 1967).

In this report we describe the application of the method to the histidine residue of lysozyme, α-lytic protease, and S.G. trypsin, as well as to the amino terminus of the latter protein. A preliminary account of this work has appeared elsewhere (Kaplan and Duggleby, 1975).

Experimental Section

Rationale. The competitive labeling method (Kaplan et al., 1971) consists of four main steps. These are: (i) treatment of a mixture of the protein under study and a suitable standard nucleophile with a trace of a radioactive electrophilic reagent; (ii) treatment of the mixture with nonradioactive reagent under denaturing conditions to render the protein and internal standard chemically homogeneous; (iii) separation, purification, and quantitation of the internal standard; and (iv) partial hydrolysis of the protein, followed by purification and quantitation of a peptide containing the residue under study. In the present procedure, several modifications have been introduced in order to simplify and generalize the individual steps.

The first step remains the same, except that due to changes in the method of quantitation, less protein is required and the selection of the internal standard to be used is not circumscribed by the need for an accurate method for chemical estimation. In the present studies, we have used L-β-imidazolelactic acid as internal standard, and [^{3}H]N_2ph-F as the labeling reagent.

In the second step we have substituted [^{14}C]N_2ph-F for nonradioactive reagent. This permits quantitation of the group under study and the internal standard by radioactivity measurements, rather than the much more laborious method of amino acid analysis previously employed (Cruickshank and Kaplan, 1972). As noted above, quantitation of the internal standard is not based on any chemical property, and a wide choice of internal standards is available. We have chosen to use L-β-imidazolelactic acid rather than *N*-acetylhistidine employed in previous studies (Cruickshank and Kaplan, 1972, 1974, 1975), as the latter gives rise to imidazolyldinitrophenylhistidine on acid hydrolysis of the N_2ph derivative. Since the protein is subjected to acid hydrolysis (see below), confusion could arise as to the source of imidazolyldinitrophenylhistidine. This difficulty does not arise when L-β-imidazolelactic acid is used as internal standard. In previous studies, complete reaction with N_2ph-F was preceded by citraconylation so that amino groups were rendered unavailable for reaction. In the present studies, we substituted acetylation since we did not require that the modification be readily reversible. The extreme insolubility of the acetylated dinitrophenylated protein expedites the separation of the protein from the internal standard. In the case of S.G. trypsin, protection of the amino groups was eliminated, as we also wished to study the properties of the amino terminus of the protein. This fully dinitrophenylated protein is also extremely insoluble.

The third step, purification and quantitation of the internal standard, was similar to that described previously (Cruickshank and Kaplan, 1972). However, carrier 2,4-dinitrophenylimidazolelactic acid was added, which facilitated detection without interfering with quantitation. As noted above, quantitation was by scintillation counting.

The final step, hydrolysis of the protein and purification of a peptide containing the residue under study, was modified to take advantage of a special feature of the proteins under study, viz. that the group being studied was the only one of its class in the protein. Acid hydrolysis of the protein yields an N_2ph amino acid which must be derived from a unique position in the protein. Since a given type of group in any protein will yield the same derivative, the purification of this derivative is the same for any protein. Thus, preliminary work of selection of an appropriate enzymic digestion procedure and establishing purification methods for the peptides generated are completely circumvented. Furthermore, the products resulting from acid hydrolysis are fewer in number and more predictable than those resulting from enzymic digests, and fewer purification steps are necessary. Quantitation of the purified N_2ph amino acid is readily achieved by scintillation counting. In experiments in which we were studying the properties of a histidine residue, carrier imidazolyl-2,4-dinitrophenylhistidine (as its *N*-acetyl derivative) was added to the protein sample to permit detection without the use of large amounts of protein. We have found imidazolyl-2,4-dinitrophenylhistidine to be quite stable to acid hydrolysis, although the literature is contradictory on this point (Hirs et al., 1965; Henkart, 1971).

Materials. S.G. trypsin and α-lytic protease were a gift from Dr. L. B. Smillie. Hen egg-white lysozyme and L-β-imidazolelactic acid were purchased from Sigma Chemical Co. Tritiated and [^{14}C]N_2ph-F were from Amersham/Searle, and nonradioactive N_2ph-F was from Aldrich. Porapak Q was supplied by Waters Associates Inc. (Milford, Mass). Other chemicals were high purity preparations from commercial sources. α-*N*-Acetylimidazolyl-2,4-dinitrophenylhistidine was prepared as previously described (Cruickshank et al., 1971).

The electrophoresis buffers employed were: pH 6.5, acetic acid–pyridine–water (3:100:900, by volume); pH 4.4, pyridine–acetic acid–water (6:10:1200, by volume); pH 3.5, pyridine–acetic acid–water (1:10:190, by volume); and pH 2.1, formic acid–acetic acid–water (1:4:45, by volume).

Trace Labeling. Lysozyme was dissolved at a concentration of 5 mg/ml in a buffer consisting of *N*-methylmorpholine (5 m*M*), acetic acid (5 m*M*), and KCl (100 m*M*), adjusted to pH 4.4 with HCl. An internal standard of L-β-imidazolelactic acid was added to a final concentration of 0.33 m*M*, and 3-ml portions were equilibrated at 20°C with a nitrogen atmosphere. The pH was adjusted as required by the addition of KOH; then 50 μl of acetonitrile containing 0.42 mCi of [^{3}H]N_2ph-F (30 Ci/mmol) was added. The reaction was allowed to proceed for 8.0 hr at 20°C, and then terminated by the addition of 1 drop of HCl.

α-Lytic protease was dissolved at a concentration of 1 mg/ml in buffer as above. Internal standard was added at a concentration of 0.20 m*M*, and 2.5-ml portions were equilibrated at 20°C. The pH was adjusted by the addition of KOH; then 50 μl of acetonitrile containing 0.50 mCi of [^{3}H]N_2ph-F (30 Ci/mmol) was added. The reaction was terminated with 1 drop of HCl after 8.0 hr incubation at 20°C.

S.G. trypsin was dissolved at a concentration of 1 mg/ml in buffer as above, but containing 15 m*M* $CaCl_2$ to stabilize the enzyme (Olafson and Smillie, 1975). In one series of experiments, the buffer also contained 5 m*M* benzamidine. Internal standard was added at a concentration of 0.20 m*M*, and 2.5-ml portions were equilibrated at 20°C. The pH was adjusted as necessary with KOH; then 50 μl of acetonitrile containing 0.23 mCi of [^{3}H]N_2ph-F (30 Ci/mmol)

Table I: Reactivity of the Histidine Residue of Hen Egg-White Lysozyme and α-Lytic Protease.

pH	α_s	$^3H_x/^{14}C_x$	$^3H_s/^{14}C_s$	αr
		Lysozyme		
4.49	0.00166	4.81	1.10	0.00725
5.00	0.00534	9.28	0.849	0.0584
5.55	0.0187	22.4	0.921	0.456
6.19	0.0768	32.7	1.40	1.79
6.80	0.253	38.6	3.11	3.14
7.38	0.563	38.1	5.67	3.78
8.00	0.843	35.4	10.6	2.82
8.65	0.960	21.8	6.39	3.28
		α-Lytic Protease		
5.04	0.00546	1.92	0.569	0.0184
5.60	0.0209	2.87	0.974	0.0617
6.23	0.0836	5.30	1.93	0.229
6.51	0.148	6.48	4.80	0.200
6.75	0.232	6.88	10.4	0.154
7.36	0.552	9.61	11.6	0.457
7.99	0.840	11.7	19.3	0.510
8.59	0.954	9.96	20.5	0.464
9.25	0.990	8.42	22.0	0.379

was added. The reaction was allowed to proceed for 6.0 hr at 20°C, and then terminated by the addition of 1 drop of HCl.

In all cases, unreacted N_2ph-F was removed by extraction with three-ml portions of diethyl ether. The ether extracts were combined, evaporated to dryness under nitrogen, dissolved in 0.5 ml of 0.01 *N* HCl, and set aside for later use.

Acetylation and Dinitrophenylation. The trace-labeled protein was denatured by the addition of urea to give a final concentration of 8 *M*. Lysozyme and α-lytic protease were acetylated by the addition of three portions of 25 μl of acetic anhydride, maintaining pH 9 using a pH-Stat. When the reaction was completed, 0.5 g of sodium bicarbonate was added, and the protein and internal standard were fully dinitrophenylated by the addition of 50 μl of [^{14}C]N_2ph-F (25 μCi for lysozyme; 12.5 μCi for α-lytic protease), diluted with an equal volume of acetonitrile. S.G. trypsin was not acetylated, and dinitrophenylation was carried out with 12 μCi of [^{14}C]N_2ph-F, in the presence of 0.3 g of sodium bicarbonate.

The reaction was allowed to proceed for 16 hr at 25°C and then terminated by lowering the pH to 2 with HCl. The protein was found to precipitate. The addition of 1 drop of octanol prior to the addition of HCl aided considerably in reducing foaming at this stage. The combined ether extracts (see above) were added;[2] then 0.5 ml of carrier 2,4-dinitrophenylimidazolelactic acid solution (see later) was added. Unreacted N_2ph-F, 2,4-dinitrophenol, and other side products were removed by extraction with 4 ml of diethyl ether, 5 to 7 times. The protein was removed by centrifugation.

Preparation of Carrier Dinitrophenylimidazolelactic Acid Solution. L-β-Imidazolelactic acid (20 mg) was dissolved in 5 ml of 8 *M* urea, and 0.5 g of sodium bicarbonate added. N_2ph-F (50 μl) was added, and the reaction allowed to proceed for 16 hr at 25°C. The solution was acidified and extracted with diethyl ether as described above and then diluted to 12 ml with water.

[2] The ether extracts were added back to avoid the objection that small amounts of [^{3}H]dinitrophenylimidazolelactic acid could be extracted into diethyl ether, thereby affecting the apparent reactivity of the internal standard.

Purification of Internal Standard. Internal standard (dinitrophenylimidazolelactic acid) was purified by ion-exchange chromatography on Amberlite IR-45 as previously described for α-*N*-acetylimidazolyldinitrophenylhistidine (Cruickshank and Kaplan, 1972). Further purification was achieved by high-voltage electrophoresis on paper at pH 6.5 ($\mu_{Dns\text{-}OH}$ = 0.87) and at pH 2.1 ($\mu_{Dns\text{-}Arg}$ = 0.66). Dinitrophenylimidazolelactic acid was located by its ultraviolet absorption.

Isolation of Imidazolyldinitrophenylhistidine. The precipitated protein was washed three times with 3-ml portions of 0.01 *N* HCl and then suspended in 1.5 ml of 6 *N* HCl. α-*N*-Acetylimidazolyldinitrophenylhistidine (1 mg for lysozyme and α-lytic protease; 0.5 mg for S.G. trypsin) was added as carrier, and the mixture hydrolysed in sealed evacuated tubes for 22 hr at 110°C. The hydrolysate was applied to a 7 × 30 mm column of Porapak Q, equilibrated with 0.01 *N* HCl. The column was washed with 5 ml of 0.01 *N* HCl, then the imidazolyldinitrophenylhistidine eluted with 3 ml of 20% (v/v) acetone. The eluate was evaporated to dryness under nitrogen; then imidazolyldinitrophenylhistidine was purified by high-voltage electrophoresis on paper at pH 3.5 ($\mu_{Dns\text{-}Arg}$ = 0.51) and pH 2.1 ($\mu_{Dns\text{-}Arg}$ = 0.79). Imidazolyldinitrophenylhistidine was located by its ultraviolet absorption.

Isolation of α-N-Dinitrophenylvaline from Dinitrophenylated S.G. Trypsin. Most N_2ph derivatives are not eluted from Porapak Q by 20% acetone. Subsequent elution with 80% (v/v) acetone will remove this material. The 80% acetone fraction was evaporated to dryness under nitrogen and then redissolved in 1 ml of 0.1 *N* HCl. α-*N*-Dinitrophenylvaline was extracted into diethyl ether; the ether extract was evaporated to dryness and dissolved in 6 *N* HCl. This material was hydrolyzed in sealed evacuated tubes for 40 hr at 110°C. This second hydrolysis step was included since the amino-terminal sequence of S.G. trypsin begins with Val-Val-Gly (Olafson et al., 1975). Extended hydrolysis ensures that any N_2ph-Val-Val is converted to α-*N*-N_2ph-Val. The hydrolysate was extracted with diethyl ether, and the extracted α-*N*-N_2ph-Val purified by high-voltage electrophoresis on paper at pH 4.4, using a Locarte flat-plate apparatus. α-*N*-N_2ph-Val was detected by its yellow color.

Radioactivity Measurements and Calculations. After elution from paper, each sample of dinitrophenylimidazolelactic acid, imidazolyldinitrophenylhistidine, and α-*N*-N_2ph-Val was dissolved in 0.2 ml of water; then 10 ml of Aquasol scintillator fluid was added. Scintillation counting was performed on a Nuclear-Chicago Isocap/300, using the program mode for unquenched ^{3}H/^{14}C samples. After correction for spillover of ^{14}C into the tritium channel, the pH-dependent second-order velocity constant relative to imidazolelactic acid (αr) at each pH value was calculated from the equation:

$$\alpha r = \alpha_s \left(\frac{^3H_x/^{14}C_x}{^3H_s/^{14}C_s} \right)$$

where α_s is the degree of ionization of imidazolelactic acid, assuming a pK_a of 7.27 (Cruickshank and Kaplan, 1975), ^3H_x and $^{14}C_x$ are the tritium and ^{14}C counts found in imidazolyldinitrophenylhistidine or in α-*N*-N_2ph-Val, and ^3H_s and $^{14}C_s$ are the corresponding counts in dinitrophenylimidazolelactic acid. As all samples were counted with approximately the same efficiency, conversion from counts per minute to decompositions per minute was unnecessary.

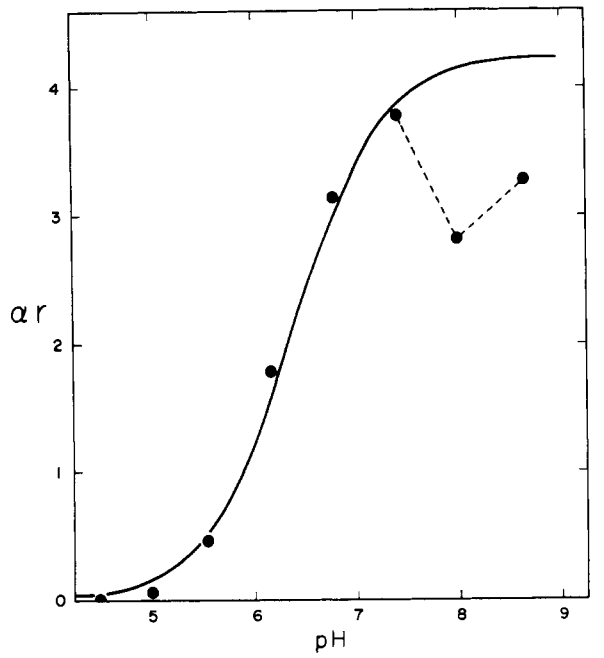

FIGURE 1: Effect of pH on the reactivity of histidine-15 of hen egg-white lysozyme. The data are taken from Table I. The solid line shows a theoretical titration curve with a pK_a of 6.4 and a reactivity of 4.25.

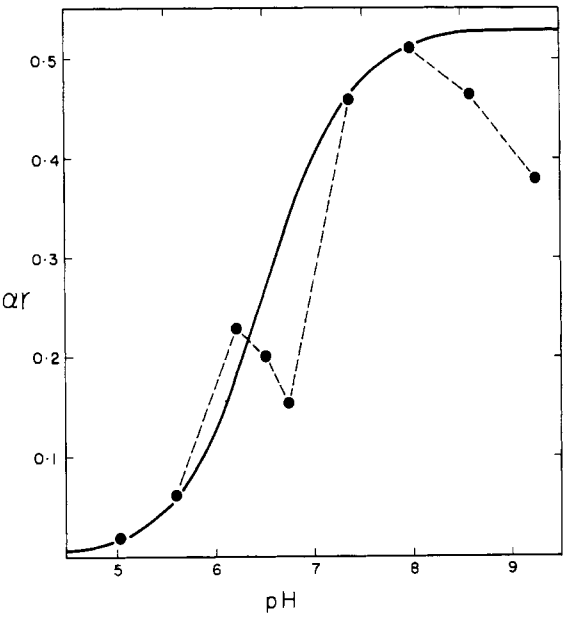

FIGURE 2: Effect of pH on the reactivity of histidine-36 of α-lytic protease. The data are taken from Table I. The solid line shows a theoretical titration curve with a pK_a of 6.5 and a reactivity of 0.53.

Results

Lysozyme. Initial studies using hen egg-white lysozyme gave the data shown in Table I. From a plot of αr vs. pH (Figure 1) it can be seen that the data below pH 7.4 closely follow a theoretical titration curve with a pK_a of 6.4 and a pH-independent second-order velocity constant of 4.25, relative to imidazolelactic acid. Given a pH-independent second-order velocity constant for the reaction of N_2ph-F with imidazolelactic acid of 0.0443 M^{-1} min^{-1} (Cruickshank and Kaplan, 1975) the velocity constant for the reaction of N_2ph-F with histidine-15 of lysozyme is 0.188 M^{-1} min^{-1}.

α-Lytic Protease. Following the initial success with lysozyme, it was clear that much smaller amounts of protein could be used. Thus, in the experiments on α-lytic protease, only 0.127 μmol of protein per labeling was used. The results obtained are shown in Table I and Figure 2. The data approximately follow a titration curve with a pK_a of 6.5 and a relative pH-independent second-order velocity constant of 0.53, corresponding to an absolute velocity constant of 0.0235 M^{-1}. However, there are two prominent deviations from this theoretical curve, one near the pK_a, and the other above pH 8.

S.G. Trypsin. S.G. trypsin, like its mammalian homolog, is inhibited by benzamidine with a K_I of approximately 10^{-5} M (R. G. Duggleby and H. Kaplan, unpublished observations; Mares-Guia and Shaw, 1965). This inhibition is undoubtedly due to occupancy of the specificity pocket, which prevents binding of the structure. It has previously been observed that occupancy of the specificity site in α-chymotrypsin, a comparable system, leads to a considerable change in the properties of the active-site histidine (Cruickshank and Kaplan, 1975). Thus, it was of interest to determine the properties of the active-site histidine of S.G. trypsin in the presence and absence of benzamidine. The results are summarized in Table II and Figure 3. In both cases, the low and high pH data follow titration curves, with a relative pH-independent second-order velocity constant of 0.74, corresponding to an absolute velocity constant of 0.0328 M^{-1} min^{-1}. The pK_a of 6.5 of the histidine is shifted to 6.8 in the presence of saturating amounts of benzamidine. At intermediate pH values, the reactivity is considerably greater than would be expected from simple titration behavior.

The properties of the amino-terminal valine of the protein were examined in the absence of benzamidine. The data (Table II and Figure 4) show that over most of the pH range examined, the α-amino group of the protein shows simple titration behavior, with a pK_a of 8.1, and a relative pH-independent second-order velocity constant of 9.1, corresponding to an absolute velocity constant of 0.403 M^{-1} min^{-1}.

Discussion

Competitive labeling is a technique which can be used to determine the reactivity and ionization constant of individual functional groups in proteins. In order to assign parameters to a specific functional group, some method of isolating this group from other reactive functional groups in the protein is necessary. This has been accomplished in previous studies using proteolytic enzymes to fragment the labeled protein into peptides which contain unique functional groups. These are then located in the primary structure of the protein from their amino acid composition or sequence. This identification procedure may be simplified in the case of a protein which contains a solitary functional group of a particular class by using a reagent which forms an acid-stable derivative with this functional group. After acid hydrolysis of the derivatized protein, the derivatized amino acid must arise from a unique position in the protein. We have chosen to use N_2ph-F as the labeling reagent as it forms acid-stable derivatives with the side chains of histidine, tyrosine, lysine, and cysteine, as well as with the α-amino group of the protein. When any of these groups is the sole representative of its type in the protein molecule, then the reactivity of this group toward N_2ph-F is readily determined by competitive labeling. In the case of the α-amino group, the method should prove particularly useful since most proteins contain a solitary group of this type. Moreover, once the purification procedure has been established

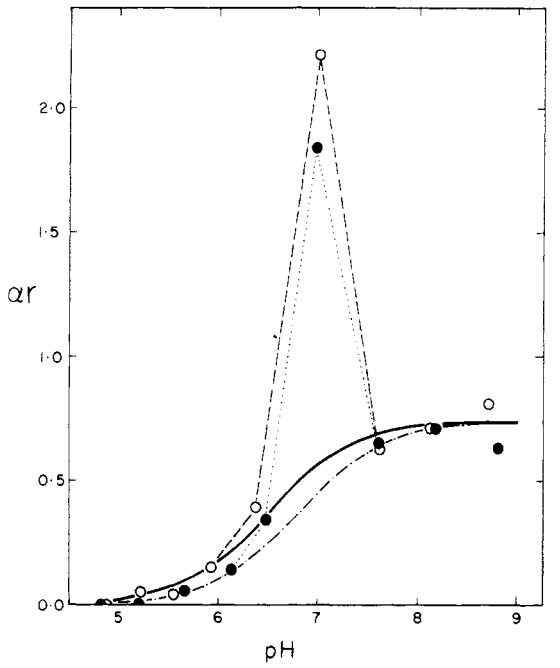

FIGURE 3: Effect of pH and benzamidine on the reactivity of histidine-37 of S.G. trypsin. The data in the presence (●) and absence (○) of benzamidine are taken from Table II. The theoretical titration curves shown have a reactivity of 0.74, and a pK_a of 6.5 (—) and 6.8 (- · -).

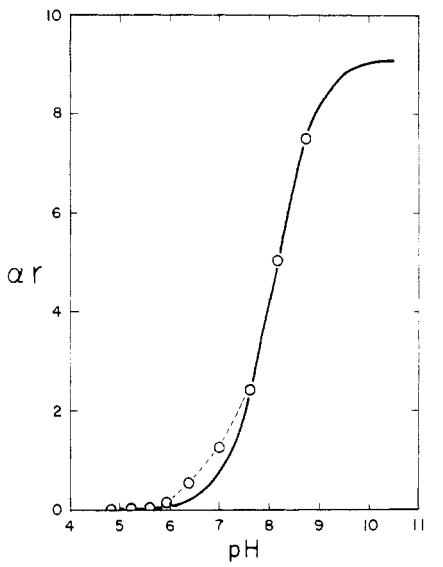

FIGURE 4: Effect of pH on the reactivity of valine-1 of S.G. trypsin. The data are taken from Table II. The solid line shows a theoretical titration curve with a pK_a of 8.06 and a reactivity of 9.14.

Table II: Reactivity of Histidine-37 and Valine-1 of S.G. Trypsin.

		$^3H_X/^{14}C_X$			αr	
pH	α_S	His-37	Val-1	$^3H_S/^{14}C_S$	His-37	Val-1
4.83	0.00362	1.48	0.501	0.998	0.00537	0.00182
5.23	0.00904	2.93	0.846	0.520	0.0509	0.0147
5.58	0.0200	2.90	1.18	1.20	0.0484	0.0196
5.94	0.0447	3.34	2.73	0.995	0.150	0.122
6.39	0.117	4.36	5.82	1.30	0.392	0.524
7.01	0.355	20.8	11.7	3.33	2.21	1.25
7.61	0.686	5.30	20.4	5.84	0.623	2.39
8.15	0.884	6.06	42.8	7.53	0.711	5.03
8.71	0.965	6.56	61.1	7.87	0.805	7.49
		5 m*M* Benzamidine				
4.83	0.00362	0.712		1.70	0.00151	
5.20	0.00844	1.11		1.12	0.00838	
5.66	0.0240	2.21		0.983	0.0539	
6.14	0.0690	2.33		1.12	0.143	
6.49	0.142	3.36		1.41	0.340	
6.98	0.339	21.8		4.02	1.84	
7.60	0.681	5.00		5.22	0.652	
8.18	0.891	5.88		7.40	0.708	
8.81	0.972	5.11		7.86	0.632	

for the α-*N*-N_2ph amino acids, imidazolyldinitrophenylhistidine, *O*-N_2ph-Tyr, ϵ-N_2ph-Lys, and *S*-N_2ph-Cys, no methodological changes are necessary in order to determine the reactivity of these residues in any protein. For example, in the present study the reactivities of the histidine residue in lysozyme, α-lytic protease, and S.G. trypsin were determined by essentially identical procedures.

An improvement over previous competitive labeling studies of histidine residues (Cruickshank and Kaplan, 1972, 1974, 1975) is the use of a double-labeling procedure. As before, reactivity is determined using [^{3}H]N_2ph-F, but complete reaction is with [^{14}C]N_2ph-F, so that reactivity determination and quantitation of the isolated derivative are performed simultaneously by scintillation counting. This procedure avoids the laborious thiolysis, hydrolysis, and amino acid analysis previously employed for quantitation, and offers additional advantages.

Since quantitation is by determination of radioactivity, and the specific radioactivity of [^{14}C]N_2ph-F may be varied at will, only small amounts of protein are required. Thus, 110 nmol of S.G. trypsin was used for each experimental point. This could easily be reduced by using [^{14}C]N_2ph-F of a higher specific radioactivity. The addition of carrier imidazolyldinitrophenylhistidine overcomes the problem of detection of small amounts of material. Another advantage of the double-labeling method is that the choice of internal standard may be based on considerations other than existence of a convenient method of chemical estimation.

The minor variations in methodology used for the three proteins are worthy of comment. In the experiments with lysozyme, we were mainly interested in establishing whether the methodology would work according to theory, and we therefore used quite large amounts of protein and internal standard (approximately 1 μmol of each). Similarly, the amounts of [^{3}H]- and [^{14}C]N_2ph-F were quite large in order to maximize incorporation of radioactivity. From our experiences with this protein, it was clear that much smaller amounts of protein could be used, and so in the experiments on α-lytic protease, the quantity of protein was reduced to one-eighth, and the quantities of internal standard and [^{14}C]N_2ph-F were reduced to one-half. The success with α-lytic protease indicated that we could, using similar quantities of protein, determine the properties of both the α-amino group and the histidine residue of S.G. trypsin in a single experiment. Consequently, the acetylation step was omitted so that [^{14}C]N_2ph-F would react with all amino groups.

The amino-terminal N_2ph amino acid was easily isolated, and the reactivity readily determined. The method may be easily adapted to any protein, although minor variations in the isolation procedure would be necessary, depending on the amino terminus of the protein. Similarly, in proteins containing a solitary lysine, tyrosine, or cysteine residue, it

should be possible to determine the reactivity of these amino acids.[3]

Thus, the present methodology may be applied to a variety of functional groups in proteins. In contrast to most chemical modification methods, the low specificity of N_2ph-F becomes an advantage. The technique is simple, rapid and sensitive, and does not require specialized equipment. We have chosen to use high-voltage electrophoresis in the final purification of the N_2ph derivatives, but chromatographic methods could be used with equal success. The method is therefore accessible to most laboratories.

Reactivity and pK_a of Histidine-15 of Lysozyme. The reactivity of histidine-15 of lysozyme closely follows a titration curve below pH 7.4, with a pK_a of 6.4 and a pH-independent second-order velocity constant of 0.188 M^{-1} min^{-1}. The titration of this residue has previously been studied by NMR spectroscopy in D_2O and has been shown to have a midpoint at a pH meter reading of 5.8 (Meadows et al., 1967) to 5.9 (Bradbury and Wilairat, 1967), corresponding to a pD of 6.2–6.3. This is close to the pK_a of 6.4 that we observe in H_2O. Studies with model compounds (Bradbury and Scheraga, 1966) have shown that the pK_a in water is lower than that observed in D_2O, but this effect in lysozyme is apparently offset by other effects of D_2O, possibly solvation effects on the protein molecule. The reactivity observed is six times greater than expected, as judged from a Bronsted plot (Bronsted and Peterson, 1924) (Figure 5). This superreactivity is discussed below. The data above pH 7.4 show a deviation from simple titration behavior. We interpret this discontinuity as indicating a conformational change in the protein.[4] Thus, it is of interest that in the alkaline pH range, NMR (Meadows et al., 1967) and other studies (reviewed by Imoto et al., 1972) have demonstrated aggregation of the protein.

Hydrogen Bonding in the Serine Proteases. It is widely held that catalysis in the serine proteases involves the cooperative interaction of an aspartic acid and a histidine residue to enhance the nucleophilicity of the active-site serine (Blow et al., 1969). X-ray crystallographic studies (see review by Blow and Steitz, 1970) have shown that the residues involved are in suitable positions and orientations to permit a hydrogen bonding network to be set up, but this does not prove the existence of such hydrogen bonds. Examination of the chemical reactivity of the N-3 position of the histidine can provide information as to the extent of hydrogen bonding. Thus, hydrogen bonding between aspartic acid and the N-1 position of the histidine should enhance the nucleophilicity at the N-3 position by donating electrons to the system. Hydrogen bonding between the N-3 position and serine should prevent the reaction of an electrophile with the histidine.

At high pH, the reactivity of the histidine of α-lytic protease (Figure 2) and S.G. trypsin (Figure 3) is close to the expected reactivity for a free histidine (Figure 5). Similarly, it has previously been shown that at high pH, histidine-57 of α-chymotrypsin behaves as a "normal" histidine

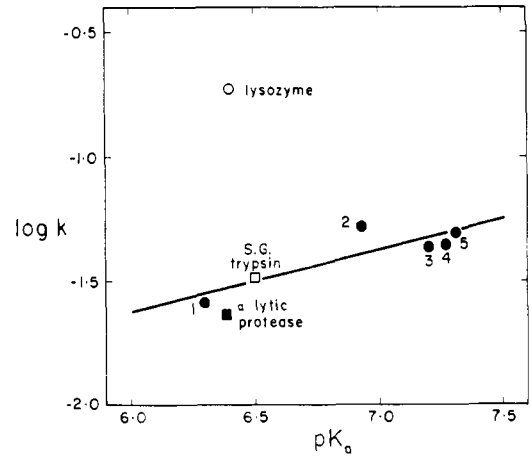

FIGURE 5: Bronsted plot for a series of imidazole compounds, and the histidine residue of lysozyme, α-lytic protease, and S.G. trypsin. The data for hydroxymethylimidazole (1), imidazole (2), α-*N*-acetylhistidine (3), L-β-imidazolelactic acid (4), and imidazoleacetic acid (5) are taken from Cruickshank and Kaplan (1975), and the line is a linear regression on these data. The parameters for lysozyme (○), α-lytic protease (■), and S.G. trypsin (□) are those used to draw the solid lines of Figures 1, 2, and 3, respectively.

(Cruickshank and Kaplan, 1972). If the aspartic-histidine-serine system of hydrogen bonding is substantially intact at high pH, then to explain the normal reactivity of the histidine, we must postulate that an equilibrium exists whereby there is a small fraction of the strongly nucleophilic form in which histidine is hydrogen bonded to aspartic acid, but not to serine. The apparently normal reactivity thus results from the fortuitous circumstance that the small fraction of the strongly nucleophilic form is exactly offset by its high reactivity. To observe such a coincidence in three proteins appears unlikely. Thus, we feel that the most plausible interpretation of the data is that histidine is not hydrogen bonded with either serine or aspartic acid at high pH.

Comparison with NMR Studies of the Serine Proteases. NMR studies have been carried out with δ-chymotrypsin and α-lytic protease (Robillard and Shulman, 1974; Hunkapiller et al., 1973). It is difficult to correlate the chemical reactivity data with the NMR data for several reasons. The NMR data closely follow theoretical titration curves and appear to be relatively insensitive to the changes in the properties of the active-site histidine observed in the present and other (Cruickshank and Kaplan, 1972) studies. This may, in part, be due to differences in the experimental conditions employed, i.e. solvent composition, and perhaps more importantly, protein concentration. Moreover, there is some confusion as to the interpretation and even the existence of certain resonances. Notwithstanding these difficulties, some comparison is possible. The pK_a of the histidine residue of these proteins obtained by NMR is similar to that observed by chemical reactivity. It is of interest that in α-chymotrypsin, histidine-57 is superreactive at pH values near neutrality. In this pH range, the resonance of the active-site histidine of chymotrypsin cannot be observed (Bradbury and Wilairat, 1967; Robillard and Shulman, 1974).

Conformational Changes in the Serine Proteases. As in α-chymotrypsin (Cruickshank and Kaplan, 1972), the active-site histidine of S.G. trypsin is much more reactive at intermediate pH values than would be predicted on the basis of simple titration behavior (Figure 3). The transition from high reactivity to normal reactivity occurs over a quite

[3] In certain instances, the requirement for solitary residues may be dispensed with. For instance, Visentin et al. (1973), studying the average reactivity of the lysine residues of each protein of the ribosome, could have used the present methodology.

[4] The concept of a conformational change is used in its broadest sense. Thus, a redistribution of electrons, whether or not this is accompanied by changes in the relative positions of the atoms of the protein, would be considered as a conformational change for the purposes of this discussion.

narrow pH range. If this transition is an equilibrium process over the time period of the trace labeling, it can be shown that at least two ionizing groups are involved. While we cannot exclude this possibility, a second alternative exists; i.e., that the system is not an equilibrium at intermediate pH values, and that the high reactivity form is in a metastable conformation. In this context, it is of interest that this high reactivity form is more apparent in α-chymotrypsin for which trace labeling was performed for 0.5 hr, than in S.G. trypsin for which trace labeling was for 6.0 hr. This is exactly what would be expected if the high reactivity form is metastable. In α-lytic protease, the reactivity of the histidine deviates only slightly from the expected titration behavior at intermediate pH values (Figure 2), which may mean that the metastable form of this enzyme is more rapidly converted to the normal reactivity form. Similarly, when the specificity site of α-chymotrypsin is occupied by indole, the high reactivity form is not observed (Cruickshank and Kaplan, 1975), suggesting that in the presence of indole, the metastable form is more rapidly converted to the normal reactivity form. Occupancy of the specificity site of S.G. trypsin does not appear to affect the stability of the high reactivity form.

While the postulation of a metastable conformation in S.G. trypsin is speculative, there is a precedent for this in chymotrypsin (Corey et al., 1965). Brief incubation of α-chymotrypsin under mildly alkaline conditions converts it to γ-chymotrypsin, which may be distinguished from α-chymotrypsin by its crystal form. Several months at mildly acidic pH are necessary to effect conversion of γ-chymotrypsin to α-chymotrypsin. Thus, at mildly alkaline pH values, α-chymotrypsin is metastable.

Properties of the Amino Terminus of S.G. Trypsin. The properties of the amino terminus of S.G. trypsin also give indications of an altered conformation of the protein near neutrality. Over most of the pH range examined the data closely resemble a titration curve, with a pK_a of 8.1 and a pH-independent second-order velocity constant of 0.403 M^{-1} min^{-1} (Figure 4). Near neutrality, the amino terminus is more reactive than would be expected from simple titration behavior.

Previous studies of the α-amino group of elastase (Kaplan et al., 1971) and the corresponding α-amino group of α-chymotrypsin (Kaplan, 1972) have shown that this group has a high pK_a and a low reactivity toward acetic anhydride, presumably because of ion-pair formation with aspartic-194 (Shotton and Watson, 1970; Matthews et al., 1967). Thus, it is surprising to find that the α-amino group of S.G. trypsin is apparently exposed on the surface of the protein. The pK_a is in the normal range expected for α-amino groups (Cohn and Edsall, 1943), and the reactivity toward N_2ph-F is similar to that observed in other proteins (Murdock et al., 1966; Hill and Davies, 1967). In the absence of a Bronsted plot for the reaction of primary amines with N_2ph-F, it is impossible to determine whether these groups are sub- or superreactive. Nevertheless, it is significant that a model valyl peptide with a pK_a of 7.9 has a velocity constant of only 0.062 M^{-1} min^{-1} at 25°C (Hill and Davies, 1967).

The ionization state of valine-1 does not appear to affect catalysis by S.G. trypsin (Olafson and Smillie, 1975). In this context, it should be noted that the ionization state of the amino terminus of δ-chymotrypsin (Valenzuela and Bender, 1969, 1970) or elastase (Kaplan and Dugas, 1969; Kaplan et al., 1971) does not appear to affect the activity of these enzymes. Olafson and Smillie (1975) were unable to substantially modify the amino terminus of S.G. trypsin, and suggested that this residue forms a buried ion pair with aspartic acid-169. Our results do not substantiate this conclusion.

Superreactivity of Histidine Residues in Proteins. As mentioned previously, hydrogen bonding between aspartic acid and the N-1 position of histidine would be expected to confer enhanced nucleophilicity on the N-3 position. It is reasonable to suppose that such a hydrogen bond is responsible for the superreactivity of the metastable form of the serine proteases. Nevertheless, in certain cases, enhanced nucleophilicity may be the result of other phenomena. Thus, histidine-40 of α-chymotrypsin is superreactive, which has been ascribed to locking of this histidine in one tautomeric form by hydrogen bonding (Cruickshank and Kaplan, 1972), and a similar phenomenon may be operating in lysozyme. It may be significant that the imidazole ring of these residues appears to be partially buried. There are no simple model systems from which one can deduce the properties of such a partially buried histidine, but we speculate that this would tend to restrict the delocalization of the N-3 electrons of the unprotonated form into the ring, thus making this nitrogen more nucleophilic.

At present the factors which affect protein conformation and the reactivity of functional groups are poorly understood. An obviously important factor is the water structure in the immediate vicinity of the protein molecule. The structure and properties of this solvation shell will undoubtedly change as the net charge on the protein is altered by titration. It is therefore not surprising that the pH–reactivity profiles reported in this paper show large deviations from ideal titration behavior. On the other hand, however, there is at present no theoretical basis by which one can satisfactorily account for these observations as due to the ionization of one or more functional groups, metastable conformational states, or the effect of solvation. Indeed, all of these possibilities may be an integral part of a highly cooperative process which involves restructuring of the protein and its solvation shell as ionizable groups on the protein are titrated.

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References

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221,* 337–340.

Blow, D. M., and Steitz, T. A. (1970), *Annu. Rev. Biochem. 39,* 63–100.

Bradbury, J. H., and Scheraga, H. A. (1966), *J. Am. Chem. Soc. 88,* 4240–4246.

Bradbury, J. H., and Wilairat, P. (1967), *Biochem. Biophys. Res. Commun. 29,* 84–89.

Bronsted, J. N., and Peterson, K. (1924), *Z. Phys. Chem. 108,* 185–235.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 84.

Corey, R. B., Battfay, O., Brueckner, D. A., and Mark, F. G. (1965), *Biochim. Biophys. Acta 94,* 535–545.

Cruickshank, W. H., and Kaplan, H. (1972), *Biochem. J. 130,* 1125–1131.

Cruickshank, W. H., and Kaplan, H. (1974), *J. Mol. Biol. 83,* 267–274.

Cruickshank, W. H., and Kaplan, H. (1975), *Biochem. J. 147,* 411–416.
Cruickshank, W. H., Radhakrishnan, T. M., and Kaplan, H. (1971), *Can. J. Biochem. 49,* 1225–1232.
Dayhoff, M. O. (1972), Altas of Protein Sequence and Structure, Washington, D.C., National Biomedical Research Foundation.
Henkart, P. (1971), *J. Biol. Chem. 246,* 2711–2713.
Hill, R. J., and Davies, R. W. (1967), *J. Biol. Chem. 242,* 2005–2012.
Hirs, C. H. W., Halmann, M., and Kycia, J. H. (1965), *Arch. Biochem. Biophys. 111,* 209–222.
Hunkapiller, M. W., Smallcombe, S. H., Whitaker, D. R., and Richards, J. H. (1973), *Biochemistry 12,* 4732–4743.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972), *Enzymes, 3rd Ed., 7,* 665–868.
Kaplan, H. (1972), *J. Mol. Biol. 72,* 153–162.
Kaplan, H., and Dugas, H. (1969), *Biochem. Biophys. Res. Commun. 34,* 681–685.
Kaplan, H., and Duggleby, R. G. (1975), *Canadian Federation of Biological Societies,* Winnipeg.
Kaplan, H., Stevenson, K. J., and Hartley, B. S. (1971), *Biochem. J. 124,* 289–299.
Mares-Guia, M., and Shaw, E. (1965), *J. Biol. Chem. 240,* 1579–1585.
Matthews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature (London) 214,* 652–656.
Meadows, D. H., Markley, J. L., Cohen, J. S., and Jardetzky, O. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1307–1313.
Murdock, A. L., Grist, K. L., and Hirs, C. H. W. (1966), *Arch. Biochem. Biophys. 114,* 375–390.
Olafson, R. W., Jurášek, L., Carpenter, M. R., and Smillie, L. B. (1975), *Biochemistry 14,* 1168–1177.
Olafson, R. W., and Smillie, L. B. (1975), *Biochemistry 14,* 1161–1167.
Olson, M. O. J., Nagabhushan, N., Dzwiniel, M., Smillie, L. B., and Whitaker, D. R. (1970), *Nature (London) 228,* 438–442.
Robillard, G., and Shulman, R. G. (1974), *J. Mol. Biol. 86,* 519–540.
Shotton, D. M., and Watson, H. C. (1970), *Nature (London) 225,* 811–816.
Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R. (1968), *J. Mol. Biol. 35,* 143–164.
Valenzuela, P., and Bender, M. L. (1969), *Proc. Natl. Acad. Sci. U.S.A. 63,* 1214–1221.
Valenzuela, P., and Bender, M. L. (1970), *Biochemistry 9,* 2440–2446.
Visentin, L. P., Yaguchi, M., and Kaplan, H. (1973), *Can. J. Biochem. 51,* 1487–1497.

Molecular Mobility and Structure of Elastin Deduced from the Solvent and Temperature Dependence of ^{13}C Magnetic Resonance Relaxation Data†

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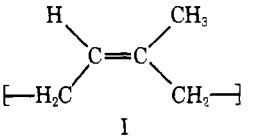
ABSTRACT: ^{13}C relaxation parameters, T_1, line width, and NOE, have been determined for backbone carbons of ligamentum nuchae elastin swollen by 0.15 *M* NaCl, 0.15 *M* NaCl–formamide, 0.15 *M* NaCl–ethanol, dimethyl sulfoxide, and formamide. The data have been analyzed in terms of (a) a single correlation time model and (b) a model employing a log-χ^2 distribution of correlation times used by Schaefer (1973) to analyze solid *cis*-polyisoprene ^{13}C relaxation data. Employing the latter mode, one obtains an approximately self-consistent quantitative analysis of all the elastin data. An average backbone correlation time, $\bar{\tau}$, of ca. 2 nsec is calculated for elastin swollen in the presence of polar organic solvents at 37°, in approximate agreement with $\bar{\tau}$ of 0.4 nsec obtained for bulk *cis*-polyisoprene at 35°. The influence of solvent and temperature on elastin spectra indicate that the larger $\bar{\tau}$ value (~80 nsec) obtained for elastin swollen by 0.15 *M* NaCl at 37° is a consequence of weak interchain polar and hydrophobic interactions, a result which is in accord with the reported viscoelastic behavior exhibited by water-swollen elastin at 37°. The results obtained further suggest that Gly, Pro, and Val residues are significantly more mobile than Ala residues, which are located in the cross-link regions. Hence, the NMR data support the view that water-swollen elastin is composed of a network of mobile chains, except possibly in the cross-link regions.

Elastin is an important component of connective tissues such as skin, lung, blood vessel, and ligament. Tissues containing elastin fibers have the useful (rubber-like) mechanical properties of high elasticity and small elastic modulus. In view of the similar mechanical properties of elastin fibers and natural rubber, a fundamental question is: does the elasticity of these two materials arise from common structural features at the molecular level?



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