Competitive Labelling, a Method for Determining the Reactivity of Individual Groups in Proteins

THE AMINO GROUPS OF PORCINE ELASTASE

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1. A method is described for determining the ionization constants and reactivities of individual amino groups in proteins. The principle is that in the presence of a trace amount of radioactive label, the various reactive groups in a protein molecule will compete for the label and the amount incorporated into any one group will be determined by its nucleophilicity, pK and micro-environment. The relative amounts of label incorporated into various groups will be proportional to their second-order rate constants and by comparing these rate constants with those expected on the basis of a linear free-energy relationship obtained with a series of standard compounds, the micro-environment can be defined for a particular amino group. 2. The method consists of treating a protein and an internal standard with a limiting amount of radioactive reagent and then with an excess of unlabelled reagent to yield a chemically homogeneous but heterogeneously labelled compound. After appropriate enzymic digestion peptides containing each labelled group are isolated and their rates of reaction, relative to the internal standard, are determined from their specific radioactivities. The entire procedure is repeated at several pH values. 3. When the method was applied to the amino groups of porcine elastase by using tritiated acetic anhydride as the labelling reagent, the *N*-terminus was found to have pK_a 9.7 and a much lower than normal reactivity. Lysine-87 and lysine-224 were found to have pK_a 10.3 and normal reactivities. At pH values greater than 10.5 there are discontinuities in all the titration curves, indicating that the entire molecule is undergoing a structural reorganization.

Since the properties of ionizable groups in proteins are sensitive to the nature of their environments, such groups can be useful built-in probes for the study of protein structure and function. The presence of charged or hydrophobic groups may shift the ionization constants of functional groups from their intrinsic values and alter the reactivity of these groups towards chemical reagents. Knowledge of the ionization constant of a particular ionizable group only partially characterizes its environment, since its dissociation constant may reflect the properties of the protein molecule as well

as the properties of the group and its environment. If, however, the reactivity of the group as well as its ionization constant are known, these two factors can be used to define the micro-environment of a particular group.

Hill & Davis (1967) and Murdock, Grist & Hirs (1966) used the fact that amino groups react with fluorodinitrobenzene only in their deprotonated form to determine the pK_a and reactivity of amino groups in haemoglobin and pancreatic ribonuclease A respectively. In both cases, dinitrophenylated peptides were isolated so that the assignment of parameters to specific residues was unequivocal. However, the methods of rate measurement and quantitative determination were not general in their applicability, but depended on unusual features of the protein molecules and the reactive

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groups under consideration. A difficulty pointed out in each instance is that the reactions were carried out under conditions in which several groups had reacted and that these modifications may have altered the reactivity of other groups. It was the object of the present investigation to develop a method that eliminates this difficulty and extends the generality of their approach. Porcine pancreatic elastase was chosen as the model protein for this study because it has only four amino groups, one of which, the N-terminal amino group, was expected to have unusual properties. Preliminary accounts of this work have been reported (Kaplan, Hartley & Stevenson, 1969; Hartley, 1970).

THEORY

Let A, B, ... N be functional groups in a protein reacting with an electrophilic reagent RX, in the presence of S, a standard nucleophile of known reactivity and pK_a . In water, the following competing reactions take place:

If the protein and standard nucleophile are in large excess over the reagent, such that the concentration of each nucleophile remains essentially constant throughout the reaction, then:

$$-\frac{d[RX]}{dt} = [RX](\sum k_A[A] + k_S[S] + k_w[H_2O] + k_{OH}[OH^-])$$

If the pH is maintained constant throughout the reaction, the function in parentheses will be a constant, k_T . Therefore:

$$-\frac{d[RX]}{dt} = k_T[RX] \text{ and } [RX] = [RX]_0 e^{-k_T t} \quad (1)$$

where $[RX]_0$ is the initial concentration of reagent.

Now:

$$\frac{d[RA]}{dt} = k_A[A][RX] = k_A[A][RX]_0 e^{-k_T t}$$

Therefore:

$$\int_0^\infty d[RA] = k_A[A][RX]_0 \int_0^\infty e^{-k_T t} \cdot dt$$

That is:

$$[RA]_\infty = \frac{k_A[A][RX]_0}{k_T}$$

where $[RA]_\infty$ is the final concentration of product.

Similarly:

$$[RS]_\infty = \frac{k_S[S][RX]_0}{k_T}$$

Therefore:

$$\frac{k_A}{k_S} = \frac{[RA]_\infty}{[A]} \bigg/ \frac{[RS]_\infty}{[S]} \quad (2)$$

If A and S are ionizing groups, then:

$$[A_T] = [A](1 + [H^+]/K_A) \text{ and } [S_T] = [S](1 + [H^+]/K_S)$$

where $[A]$ and $[S]$ are the concentrations of the nucleophilic species; $[A_T]$ and $[S_T]$, the total concentration of all ionizing species and K_A and K_S , the acid dissociation constants. Hence:

$$\frac{[RA]_\infty}{[A_T]} \bigg/ \frac{[RS]_\infty}{[S_T]} = \frac{k_A}{(1 + [H^+]/K_A)} \bigg/ \frac{k_S}{(1 + [H^+]/K_S)} = \frac{\alpha_A k_A}{\alpha_S k_S} \quad (3)$$

where $\alpha_A = 1/(1 + [H^+]/K_A)$ and $\alpha_S = 1/(1 + [H^+]/K_S)$.

If, as in the following experiments, a radioactive reagent is used in the competitive labelling and the protein and standard are subsequently made to react fully with non-radioactive reagent, $[RA]_\infty/[A_T]$ and $[RS]_\infty/[S_T]$ will be the specific radioactivities of RA and RS. Hence:

$$\frac{\text{Specific radioactivity of RA}}{\text{Specific radioactivity of RS}} = \frac{\alpha_A k_A}{\alpha_S k_S} \quad (4)$$

By measuring these specific radioactivities for groups labelled at a series of different pH values, we can calculate k_A and K_A , knowing k_S and K_S . If k_S is not known, the ratio of the velocity constants, $r = k_A/k_S$, is a useful measure of the reactivity of the group in the protein compared with that of a simple un-ionized amine in free solution, i.e.:

$$\alpha r = \alpha_S \times \frac{\text{Specific radioactivity of RA}}{\text{Specific radioactivity of RS}} \quad (5)$$

MATERIALS

Enzymes. Porcine elastase was prepared from Trypsin 1-300 (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) by the method of Lewis, Williams & Brink (1956) as modified by Smillie & Hartley (1966). Bovine pepsin, trypsin and chymotrypsin were crystalline salt-free preparations obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Chemicals. All amino acids and peptides were high-purity preparations obtained from commercial sources and were used without further purification. $[1\text{-}^{14}\text{C}]$ Acetic anhydride (specific radioactivity 45.1 mCi/mmol) and $[3\text{H}]$ acetic anhydride (specific radioactivity 3.57 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[1\text{-}^{14}\text{C}]$ Acetic anhydride (specific radioactivity 81.5 mCi/mmol) was obtained from Amersham/Searle Corp., Des Plaines, Ill., U.S.A.

METHODS

Labelling procedure. A sample (25 mg; 1 μ mol) of porcine elastase and 1 μ mol of L-phenylalanine were dissolved in 2.5 ml of 0.10M-KCl. The reaction mixture, which was contained in a water-jacketed, nitrogen-flushed cell maintained at $10.0 \pm 0.05^\circ\text{C}$, was allowed 5–10 min to achieve temperature equilibrium. The pH was adjusted to the desired value with 2M-KOH and 50 μ l of acetonitrile containing 0.7 μ mol of $[3\text{H}]$ acetic anhydride (specific

radioactivity 3.57 Ci/mmol) was added. Vigorous stirring was maintained throughout by a Radiometer type SMP1a stirring motor. The reaction mixture was left for 10 min at a constant pH value. The pH was lowered to 2 with 1 M-HCl and 2 g of urea was added to make the final solution 8 M in urea. After 15 min the solution was adjusted to pH 7 with 2 M-KOH and the elastase was completely acetylated by the addition of five 20 μ l samples of unlabelled acetic anhydride at 5 min intervals. The solution was maintained at pH 7 by means of a pH-stat. After complete reaction, the solution was adjusted to pH 2 with conc. HCl.

Purification of acetylphenylalanine. The reaction mixtures were extracted three times with 5 ml of ethyl acetate and extracts were evaporated to dryness in a rotary evaporator. The dried extracts were dissolved in 1 ml of 20 mM-NH₃ and spotted out along a 10 cm band at a position 15 cm from the cathode end of an 80 cm strip of Whatman 3MM paper. Flat-plate ionophoresis was carried out at pH 6.5 for 2 h at a voltage gradient of 60 V/cm. A 2 cm band with a mobility of 0.8 relative to aspartic acid (determined by radioautography with [¹⁴C]-acetylphenylalanine) was cut out from the dried ionogram and eluted with 20 mM-NH₃.

Digestion of labelled protein. Some acetylated elastase precipitates at the interphase during extraction with ethyl acetate. The precipitated elastase and the aqueous phase were dialysed thoroughly against water and then freeze-dried. The following sequential enzymic digests were carried out at 37°C: pepsin (0.5 mg) in 10 ml of 10% (v/v) formic acid for 12 h and then freeze-dried; trypsin (0.25 mg) in 10 ml of 1% (w/v) NH₄HCO₃ for 8 h; chymotrypsin (0.25 mg) in the same solution for 12 h and then freeze-dried.

Purification of labelled peptides. The freeze-dried peptic-tryptic-chymotryptic digests of acetylated elastase were dissolved in 2 ml of 20 mM-NH₃ and then spotted out along a 25 cm band in the centre of a Whatman 3MM sheet. Ionophoresis at pH 6.5 was carried out for 1 h at 60 V/cm. The resulting radioactive acidic, neutral and basic peptides were further purified by a combination of chromatography in butanol-acetic acid-water-pyridine (15:3:12:10, by vol.) (solvent BAWP) and ionophoresis at pH 3.5 and 2.1.

To detect the positions of ³H-labelled peptides and peptides which may not have incorporated any radioactive label, elastase in 8 M-urea was acetylated with [¹⁴C]acetic anhydride. The [¹⁴C]acetylated elastase was then passed through the same digestion procedure as the ³H-labelled material and spotted as a 1 cm marker band alongside each end of the 25 cm band. In going from one purification procedure to the next, the ¹⁴C-labelled spots were cut out along with the entire band and served as the markers for the next step. After the final purification step, the ¹⁴C-labelled material at either end of the strip was removed and the remainder of the band eluted.

Competitive labelling of a mixture of peptides and amino acids. Samples (500 nmol) of peptide or amino acid and of L-phenylalanine were dissolved in 2.50 ml of 0.10 M-KCl solution. The reaction mixture was continuously flushed with N₂ and maintained at 10.0 $\pm$ 0.05°C. The pH was adjusted to 12 with 2 M-KOH and 75 nmol of [1-¹⁴C]acetic anhydride (specific radioactivity 81.5 mCi/mmol) in 25 μ l of Spectrograde acetonitrile was added. Vigorous stirring

was obtained by means of a disc-shaped magnetic stirrer. After 5 min, 100 μ l of unlabelled acetic anhydride was added and the pH maintained at 8 with a pH-stat. When acetylation was completed, the pH was adjusted to 2 with conc. HCl.

Purification of labelled peptides and amino acids. The reaction mixtures were evaporated to dryness, and evaporation was repeated twice after additions of 5 ml of 10% (v/v) acetic acid. The residues were dissolved in 1 ml of water and the pH adjusted to 7 with 0.1 M-NaOH. The mixture was transferred with two 1 ml rinses to a column (10 cm $\times$ 0.9 cm) of Dowex 50W (X2; 200-400 dry mesh) in the H⁺ form. The column was eluted with water and the first 35 ml of effluent was collected. The effluents were evaporated to dryness, dissolved in 1 ml of 20 mM-NH₃ and spotted out along 10 cm of Whatman 3MM paper, 15 cm from the cathode end. Flat-plate ionophoresis was carried out for 2 h at 60 V/cm and pH 6.5. The dried ionograms were radioautographed for 72 h and the radioactive bands were eluted with 20 mM-NH₃. Acetylglutaminyglycine did not separate from acetyl-L-phenylalanine at pH 6.5 and was re-run at pH 3.5.

Preparation of N-terminal peptide from elastase. The N-terminal peptide Val-Val-Gly-Gly-Thr-Glu (Shotton & Hartley, 1970) was prepared by digesting 200 mg of elastase with 4 mg of pepsin in 10 ml of 1.3 M-formic acid for 16 h at 37°C. The peptide was purified by chromatography on Sephadex G-25 (fine grade) in 20 mM-acetic acid (elution vol./total bed vol. ratio 0.68) and paper ionophoresis at pH 6.5 (mobility 0.44 relative to aspartic acid).

Competitive labelling of Val-Val-Gly-Gly-Thr-Glu. Portions (500 nmol) of the N-terminal peptide Val-Val-Gly-Gly-Thr-Glu and of L-phenylalanine were dissolved in 2.5 ml of 0.1 M-KCl and the pH was adjusted to 12 with 2 M-KOH. Then 0.7 μ mol of [³H]acetic anhydride (specific radioactivity 3.57 Ci/mmol) in 50 μ l of acetonitrile was added. After 10 min, 100 μ l of unlabelled acetic anhydride was added and the pH maintained at 8 with a pH-stat until reaction was complete.

Separation and purification of acetyl-Val-Val-Gly-Gly-Thr-Glu and acetylphenylalanine. The reaction mixture was adjusted to pH 4 and then evaporated to dryness. The residue was dissolved in 20 mM-NH₃ and applied to a Sephadex G-25 (fine grade) column (50 cm $\times$ 1 cm) and 1.0 ml fractions were collected. A portion (10 μ l) was taken from each tube for scintillation counting. Tubes 19-21 were pooled for the acetylated N-terminal peptide and tubes 39-46 for acetylphenylalanine, and evaporated to dryness. The residue from tubes 39-46 was dissolved in 3 ml of water, adjusted to pH 2, extracted with ethyl acetate and the extract evaporated to dryness. Both fractions were spotted out along 10 cm of Whatman 3MM paper and subjected to flat-plate ionophoresis at pH 6.5, 60 V/cm.

Measurements of pH and pH-stat operation. A Radiometer pH-meter type TTT 1c fitted with a type PHA 924 manual temperature compensator, a type G 202C glass electrode and a type K 401 calomel reference or a Radiometer pH-meter 26, Titrator 11, type G 2222C and K 4112 electrodes were used for pH measurements and pH-stat titrations.

Rate of acetylation of phenylalanine. This was followed by mixing 0.5 ml of 20 mM-acetic anhydride in dry

acetonitrile with 25 ml of phenylalanine (2.0, 8.0 or 9.1 mM) in 0.1M-KCl at $10.0 \pm 0.1^\circ\text{C}$ and titrating at constant pH with 0.10M-NaOH in the pH-stat. Controls of 0.5 ml of 20 mM-acetic anhydride plus 25 ml of 0.1M-KCl were used to determine the spontaneous hydrolysis of the reagent at each pH value. Half-lives were determined from Guggenheim (1926) plots of the NaOH-titration results, which were in all cases first-order.

Amino acid analyses. Amino acid analyses were carried out in a Beckman-Spinco model 120 or a Beckman 120C amino acid analyser by the procedure of Spackman (1963). Acetylated peptides and amino acids were hydrolysed in 5.7M-HCl at 110°C in sealed evacuated tubes.

Scintillation counting. Scintillation counting was carried out with a Nuclear-Chicago Unilux I or Beckman LS-250 liquid-scintillation counter, with toluene-2-methoxyethanol (3:1, v/v) containing 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (7g/l) and 2,5-diphenyloxazole (0.25g/l) as the scintillator fluid.

High-voltage ionophoresis. The apparatus described by Brown & Hartley (1966) was used for acetylated polypeptides. For acetylated dipeptides and amino acids, a Locarte flat-plate apparatus was used.

Radioautography. Several dots were made with radioactive ink on the papers, which were taped with an X-ray film in cardboard folders and stored under even pressure. After development the dots help to align the film with the original paper.

RESULTS

Competitive labelling of the internal standard and protein. One uncertainty in using a reactive reagent such as acetic anhydride is how effectively the protein and internal standard will compete with water and OH^- ions for the radioactive label. Fig. 1 shows that even at high pH values a substantial portion of the radioactivity is incorporated into the protein and internal standard. A temperature of 10°C was chosen so as to minimize thermal denaturation and autodigestion during the reaction which, owing to the high reactivity of acetic anhydride, has for all practical purposes gone to completion in less than 10 min. Since a small amount of radioactive reagent is added, the buffering capacity of the protein and internal standard is sufficient to prevent any measurable change in pH. Although in principle it is possible to use quite different concentrations of protein and internal standard (see the Theory section), the use of the same concentration (0.4 mM in the experiments described) will increase the chances of obtaining products that do not differ greatly in yield and specific radioactivity.

Digestion of protein and purification of peptides. The main difficulty encountered in obtaining a suitable digest was the elimination of insoluble 'cores'. Peptic digestion of acetylated elastase gave products that were soluble in 10% (v/v) formic acid. Although the products of peptic digestion were not completely soluble in pH 6.5 buffer, further diges-

tion with trypsin, followed by chymotrypsin, yielded products that were completely soluble.

Plate 1 shows the results of radioautography of a peptic-tryptic-chymotryptic digest of elastase, acetylated in 8M-urea with $[^{14}\text{C}]$ acetic anhydride, after high-voltage ionophoresis at pH 6.5 and chromatography in solvent BAWP of the acidic and basic peptides. Peptides A1 and A3 were ninhydrin-negative and gave the following compositions after 24h hydrolysis. Peptide A1: Thr (1.0), Glu (2.0), Gly (2.3), Ala (1.2), Val (1.3), Arg (1.0); peptide A3: Thr (1.0), Glu (1.0), Gly (2.0), Val (1.4). Both peptides gave 2 mol of valine/mol of peptide after 72h hydrolysis. From the known sequence (Shotton & Hartley, 1970) these peptides correspond to the N-terminal peptides:

Peptide A1: acetyl-Val-Val-Gly-Gly-Thr-Glu-Ala-Gln-Arg

Peptide A3: acetyl-Val-Val-Gly-Gly-Thr-Glu

Peptide A1 was obtained in higher yield and was used for quantitative determination of the reactivity of the N-terminal residue.

Plate 2 shows the results of high-voltage ionophoresis at pH 2.1 of the neutral band (Plate 1) followed by chromatography in solvent BAWP. The major radioactive bands were still impure after chromatography in solvent BAWP and were further purified by high-voltage ionophoresis at pH 3.5. Two ninhydrin-positive peptides were obtained, which after 24h acid hydrolysis gave the following amino acid compositions. Peptide N4: Thr (1.0), Pro (1.0), Val (1.0), Phe (1.0), Lys (0.9); peptide N6: Glu (1.0), Val (1.0), Lys (0.9). Use of the dansyl-Edman procedure (Gray, 1967) gave the following sequences for the peptides:

Peptide N4: Lys(Ac)-Pro-Thr-Val-Phe

Peptide N6: Val-Glx-Lys(Ac)

Since the latter was neutral at pH 6.5 it must be Val-Gln-Lys(Ac). From the known sequence these peptides contain lysine-224 and lysine-87 respectively. The numbering used throughout this paper is that of chymotrypsinogen A (Shotton & Hartley, 1970).

Peptide B1 (Plate 1) was not pure after chromatography in solvent BAWP but contained, in poor yield, a peptide with lysine-87. All other radioactive bands contained peptides in too low a yield for identification.

Since there is the possibility that very little radioactivity will be incorporated into some groups of interest on the protein, direct radioautography of digests from competitively labelled protein is not a suitable method for locating peptides. Radioautography can be used, however, if a similar digest, in which the groups of interest have been separately labelled with ^{14}C , is spotted alongside the digest

EXPLANATION OF PLATE I

High-voltage ionophoresis at pH 6.5 and chromatography in butanol-acetic acid-water-pyridine (solvent BAWP) of peptides from the peptic-tryptic-chymotryptic digest of elastase acetylated in 8M-urea with [^{14}C]acetic anhydride.

EXPLANATION OF PLATE 2

High-voltage ionophoresis at pH 2.1 and chromatography in solvent BAWP of neutral peptides from Plate 1.

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EXPLANATION OF PLATE 3

Use of ^{14}C -labelled marker digest to locate ^3H -labelled peptides after high-voltage ionophoresis at pH 2.1.

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Fig. 1. Incorporation of $[^3\text{H}]$ acetic anhydride into elastase (●) and phenylalanine (○). The labelling was performed as described in the text.

from the competitively labelled protein. Plate 3 demonstrates the use of this technique to locate the peptides after high-voltage ionophoresis at pH 2.1 (cf. Plate 2). In Plate 3, some slight radioautography of the ^3H -labelled material between the two markers can be seen for peptides N4 and N6.

The acetylphenylalanine is easily separated from the protein and salts by extraction with ethyl acetate. A final purification by flat-plate ionophoresis is carried out to remove any acetylated peptides, other acetylated amino acids and $[^3\text{H}]$ -acetic acid which may have been extracted.

Identical fractionations of each acetylated peptide and the acetylphenylalanine were made for competitive labellings carried out over a range of pH values. Scintillation counting and amino acid analysis gave the specific radioactivities of each, which were used to calculate the rate constants from eq. (4) above, as shown in Table 1. The degree of dissociation of phenylalanine (α_s) was calculated by assuming $pK_s = 9.50$ at 10°C , from the value of 9.13 at 25°C and $\Delta H = 10\text{kcal/mol}$ given by Cohn & Edsall (1943).

Determination of the absolute rate of reaction of phenylalanine with acetic anhydride at 10°C . For most of the purposes of this study it was sufficient

to determine r , the rate of reaction of the amino group in the protein relative to that of phenylalanine. However, to facilitate comparison with more conventional kinetic methods, the velocity constant for reaction of phenylalanine with acetic anhydride under comparable conditions was determined separately.

Mixtures of phenylalanine and acetic anhydride in 0.1M-potassium chloride–2% acetonitrile, at 10°C , were titrated with 0.1M-sodium hydroxide in the pH-stat at various pH values between 6.4 and 7.1. From the slopes of the first-order plots the half-lives ($t_{1/2}$) were determined as shown in Table 2 and k_s was calculated from the equation:

$$\alpha_s k_s [\text{Phe}] + k_w = 2.3 \log (2/t_{1/2})$$

where α_s was calculated by assuming $pK_s = 9.50$ as above, and k_w was determined from the spontaneous hydrolysis rates. A mean value for k_s of $4.4(\pm 0.3) \times 10^4 \text{M}^{-1} \cdot \text{min}^{-1}$ was found for reaction under these conditions. Brouwer, Van der Vlugt & Havinga (1958) found for the reaction of acetic anhydride at 0°C with serine ($pK_a = 9.15$ at 25°C) that $k = 2.7 \times 10^4 \text{M}^{-1} \cdot \text{min}^{-1}$, equivalent to about $5 \times 10^4 \text{M}^{-1} \cdot \text{min}^{-1}$ at 10°C . A value of $k_s = 4.4 \times 10^4 \text{M}^{-1} \cdot \text{min}^{-1}$ for the reaction of acetic anhydride

Table 1. *Competitive labelling of porcine elastase with [³H]acetic anhydride at 10°C in 0.1 M-potassium chloride*

The specific radioactivities are of peptides or acetylphenylalanine isolated after competitive labelling at the pH indicated. α_s is the degree of dissociation of the amino group of phenylalanine, calculated assuming $pK_a = 9.50$ at 10°C, and α that of the appropriate amino group in the protein. $r = k/k_s$ is the ratio of the second-order velocity constants for reaction of the unprotonated amino group with acetic anhydride at 10°C (k) compared with that for unprotonated amino group of phenylalanine (k_s).

pH	<i>N</i> -Acetylphenylalanine		Peptide A1		Peptide N4		Peptide N6	
	$10^{-6} \times$ Radio-activity (c.p.m./ μ mol)	α_s	$10^{-5} \times$ Radio-activity (c.p.m./ μ mol)	$10^2 \times \alpha r$	$10^{-6} \times$ Radio-activity (c.p.m./ μ mol)	αr	$10^{-6} \times$ Radio-activity (c.p.m./ μ mol)	αr
8.35	5.88	0.0660	2.16	0.242	0.714	0.00799	0.967	0.0108
8.78	7.86	0.160	3.08	0.627	1.01	0.0205	1.53	0.0312
9.20	11.0	0.333	3.10	0.939	2.69	0.0816	3.98	0.121
9.60	12.0	0.557	3.86	1.79	7.73	0.359	10.5	0.487
10.10	9.37	0.799	3.58	2.98	13.3	1.13	16.1	1.37
10.40	9.07	0.888	3.72	3.64	15.7	1.54	17.6	1.72
10.82	7.72	0.954	5.47	6.76	21.0	2.59	19.3	2.39
11.21	5.50	0.981	5.75	10.3	19.1	3.40	16.3	2.90
11.60	3.37	0.992	6.85	20.1	18.8	5.53	15.7	4.62

Table 2. *Reaction of acetic anhydride with phenylalanine in 2% acetonitrile-0.1 M-potassium chloride at 10°C*

See the text for details and definitions of symbols.

pH	Phenylalanine added (mM)	$t_{\frac{1}{2}}$ (min)	k_w (min ⁻¹)	$10^3 \times \alpha_s$	$10^{-4} \times k_s$ (M ⁻¹ · min ⁻¹)
6.41	0	11.3	0.061	0.81	4.6 ± 0.3 (mean of 6)
	1.75	5.7	0.061	0.81	
	7.50	2.0	0.061	0.81	
	8.64	1.9	0.061	0.81	
6.70	0	11.1	0.062	1.59	4.3 ± 0.3 (mean of 3)
	7.50	1.2	0.062	1.59	
7.04	0	10.8	0.064	3.45	4.1 ± 0.1 (mean of 3)
	1.82	2.13	0.064	3.45	
	7.50	0.62	0.064	3.45	

with phenylalanine is used throughout this paper to allow absolute calculations of other velocity constants.

N-Terminal amino group (valine-16) (peptide A1). Fig. 2 shows a plot of αr against pH for the *N*-terminus of elastase. The solid line is a theoretical curve for $r = 0.044$ and $pK_a = 9.7$. The closed circles are experimental points. Above pH 10.5 there is a discontinuity in the results, with the reactivity of the *N*-terminus increasing sharply. The linear plot of the results in Fig. 3 emphasizes the fact that the points above pH 10.5 do not fit the same titration curve, since:

$$\alpha r = r/(1 + [H^+]/K_a) = r - \alpha r[H^+]/K_a$$

Fig. 2. Plot of αr against pH for the *N*-terminal valine residue of elastase. The solid line is a theoretical curve for $r = 0.044$ and $pK_a = 9.7$.

Fig. 3. Plot of $\alpha r \cdot [H^+]$ against αr for the *N*-terminus of elastase.

Reactivity of the amino group in the peptide Val-Val-Gly-Gly-Thr-Glu. In view of the apparently low reactivity of the terminal amino group in native elastase, the reactivity of an *N*-terminal peptide from elastase was determined. The results of competitive labelling at pH 12 are as follows: the specific radioactivity of the acetylphenylalanine was 12.5×10^6 c.p.m./ μ mol, and of the peptide was 5.93×10^6 c.p.m./ μ mol. Therefore, r for the peptide is 0.475. This value is greater than the high pH values for the terminal amino group (Fig. 2) and indicates that at high pH values the reactivity of the terminal amino group of elastase is approaching the reactivity expected from a peptide.

Lysine-224 (peptide N4). Fig. 4 is a plot of the results for lysine-224. The solid line is a theoretical curve for $r = 2.5$ and $pK_a = 10.3$. Again a discontinuity is evident in the results at pH 10.5; however, the increase in reactivity is not as marked as for the terminal amino group.

Lysine-87 (peptide N6). Fig. 5 is a plot of the results for lysine-87. The solid line is a theoretical curve for $r = 3.0$ and $pK_a = 10.3$. There is again a discontinuity above pH 10.5 but it is not as marked as for the terminal amino group and lysine-224.

'Relative reactivity' of the amino groups of elastase. The reactivity of a series of simple primary amines with acetic anhydride should parallel their basicity in a linear free-energy relationship (Leffler & Grunwald, 1963) exemplified in Fig. 6, which follows the Brønsted equation:

$$\log k = \alpha + \beta \cdot pK_a$$

where α and β are constants. To compare the reactivities of amino groups in proteins with those

Fig. 4. Plot of αr against pH for lysine-224 of elastase. The solid line is a theoretical curve for $r = 2.5$ and $pK_a = 10.3$.

of simple unhindered primary amines, we propose to define the term 'relative reactivity' as k/k_0 , where k is the observed second-order velocity constant for the amino group and k_0 is that expected from the observed pK_a according to the Brønsted equation.

It was therefore necessary to determine the reactivity of a series of simple amines under our 'competitive labelling' conditions. A mixture of amines of known pK_a values together with phenylalanine was competitively labelled with $[1-^{14}C]$ acetic anhydride at pH 12 as described above, and the velocity constants were calculated from the specific radioactivities of the acetylated products according to eqn. (4), by using the value of $k_s = 44000 \text{ M}^{-1} \cdot \text{min}^{-1}$ for phenylalanine. Fig. 6 shows the Brønsted plot for these compounds, which obeys the equation:

$$\log k = 0.46(\pm 0.03) pK_a - 4.5(\pm 0.1)$$

Brouwer *et al.* (1958) report a Brønsted slope of approximately 0.5 for reaction of primary amines

Fig. 5. Plot of αr against pH for lysine-87 of elastase. The solid line is a theoretical curve for $r = 3.0$ and $pK_a = 10.3$.

with acetic anhydride at 0°C. The velocity constants for reaction of acetic anhydride with the amino groups of native elastase are also shown in Fig. 6, and the 'relative reactivity' of these groups is shown in Table 3. Lysine-87 and lysine-224 have normal reactivity and pK_a values, indicating that they are freely accessible to reagent in solution, whereas the *N*-terminal group (valine-16) is 'buried' with very low relative reactivity.

DISCUSSION

Principle of competitive labelling. The reaction of an electrophilic reagent with groups in aqueous solution consists of a series of reactions between nucleophiles such as amino groups, phenolic groups, water and OH^- ion, which compete for the reagent. The relative rates of these reactions will depend on the nucleophilicity of the group, the ionization constant and the micro-environment in the protein. If these relative rates can be determined as a

function of pH, the latter two factors can be defined for a particular class of nucleophile. A legitimate objection to the use of reagents to explore the chemical reactivity of groups in proteins is that substitution of one group may well affect the reactivity of neighbouring groups. However, so long as the fraction of any particular group which has reacted is negligible in comparison with the total amount of unsubstituted group, the reagent is always reacting predominantly with the unsubstituted protein and this objection no longer applies.

Acetic anhydride is a suitable reagent for such a study because it is small and therefore likely to be accessible to a large number of groups, and uncharged so as to avoid electrostatic effects. Since the aim of the study is to explore the reactivity of groups in the native protein, it is desirable that the reagent should react very rapidly, and acetic anhydride fulfils this requirement. By using reagent of high specific radioactivity, it is possible to achieve accurately measureable values at extremely low degrees of labelling of any group, as required above.

Under the conditions used, the ratios of the final specific radioactivities of the groups that have reacted are proportional to the second-order velocity constants (αk) for the reaction at any particular pH value (see the Theory section). We know that charged amino groups show negligible reactivity towards electrophiles, and therefore the pK_a and reactivity of the deprotonated group can be calculated from the pH dependence of αk . This can easily be determined by including with the protein in the reaction mixture an internal standard whose absolute reactivity can be separately measured. A suitable internal standard should not have any strong specific interactions with the protein and be sufficiently reactive under the conditions used to yield products of measurable specific activity which can be readily separated and analysed. Phenylalanine was chosen for this study because the acetylphenylalanine could be separated from the protein by extraction and determined by amino acid analysis after acid hydrolysis. Other internal standards may well be more suitable for other proteins and other procedures.

After reaction of the protein with a trace amount of radioactive reagent, a chemically heterogeneous group of molecules has been formed. These can be converted into a heterogeneously labelled chemically homogeneous compound by reaction with an excess of unlabelled reagent. In this study, the reaction with unlabelled acetic anhydride was carried out in 8M-urea since 'buried' groups would thereby become accessible.

The isolation and purification of peptides derived from each individual group is clearly the major problem in such a study. The appropriate con-

Fig. 6. Brønsted plot for the reaction of acetic anhydride with amines and native elastase at 10°C in 0.1 M-KCl. r is the ratio of the second-order velocity constant for the unprotonated amino group (k) to that for unprotonated phenylalanine (k_s). k is calculated by using a value of $k_s = 44000 \text{ M}^{-1} \cdot \text{min}^{-1}$.

Table 3. Relative reactivity of amino groups in porcine elastase with acetic anhydride at 10°C

$r = k/k_s$ is the ratio of the second-order velocity constant for reaction of the unprotonated amino group (k) to that for unprotonated amino group of phenylalanine (k_s). Values of k are calculated by using $k_s = 44000 \text{ M}^{-1} \cdot \text{min}^{-1}$. 'Relative reactivity' is the ratio of k to that predicted for a normal amine (k_0) from the Brønsted plot (Fig. 6).

Amino group	r	$k (\text{M}^{-1} \cdot \text{min}^{-1})$	pK_a	Relative reactivity
Valine-16	0.044	1900	9.7	0.04
Lysine-224	2.5	110000	10.3	1.3
Lysine-87	3.0	130000	10.3	1.6

ditions can readily be explored by treating the protein with ^{14}C -labelled reagent. Paper-separation techniques and radioautography can be used as rapid probes for an appropriate purification. Column purifications are not particularly suitable for this purpose because of the number of parallel separations which are ultimately required. Any digestion can be used, but the aim is to achieve a simple purification in reasonable yield of one peptide containing each unique group. An initial peptic digestion has the advantage that insoluble 'cores' will rarely be formed. Subsequent digestion by trypsin, chymotrypsin, papain, thermolysin etc.

may generate suitable peptides that are easier to purify.

For the actual study of the reactivity ^3H -labelled reagents are preferable since they can be obtained with very high specific radioactivities. Even though these are less suitable for radioautography, this technique can still be used to locate the desired peptides if samples of an identical digest of the completely ^{14}C -labelled protein are used as markers (Plate 3). In this way groups of extremely low reactivity, such as the N -terminal residue of elastase, can be investigated (Fig. 2).

Reactivity of groups in proteins. The extent to which a group in a protein reacts with a particular reagent depends on the nucleophilicity and pK_a of the group and the 'steric' factors contributed by the protein. By measuring both the pK_a for the group and the velocity constant for the nucleophile, the steric factors can be distinguished. The pK_a is a property of the group and its environment and is the same for all reagents. It is now possible, for example, to predict the extent to which any of the amino groups studied in elastase should react with a electrophilic reagent. The size, shape and charge of the reagent will, however, influence the reaction rate and these will be reflected in the velocity constant for the amino group.

It was expected that a plot of $\log k$ versus pK_a for the reaction of a series of simple amines with acetic

anhydride would show a good linear relationship and Fig. 6 indicates that this is the case. Steric hindrance for a 'buried' amino group will be reflected in a point which lies below this line. A point above this line indicates an abnormally high reactivity, such as might result from a specific binding or orientation of the reagent at the protein surface. A low relative reactivity (see the Results section) is therefore a numerical measure of a 'buried' group and a relative reactivity much greater than 1 is the definition of a 'specific' reaction.

Amino groups of porcine elastase. The amino groups of lysine-87 and lysine-224 have normal reactivity and pK_a values slightly lower than that expected, 10.8, for a normal amino group at 10°C (Tanford, 1962), suggesting that they lie in the surface of the native protein, freely accessible to reagent. However, Fig. 2 demonstrates that the *N*-terminus of elastase has pK_a (app.) 9.7. A typical *N*-terminal group would have pK_a 7.5 at 25°C (Tanford, 1962), equivalent to pK_a 7.9 at 10°C, so that this is an abnormally high value. It has been shown for both chymotrypsin and elastase (Birktoft, Blow, Henderson & Steitz, 1970; Shotton & Watson, 1970) that the corresponding *N*-terminal groups are involved in an electrostatic interaction with aspartic acid-194 in an internal 'pocket' of the native structure, which would be expected to increase the pK_a of the amino group. A pK_a (app.) of approx. 8.5 for this group in chymotrypsin has been postulated by Hess, McConn, Ku & McConkey (1970) to account for variations in optical rotation between native chymotrypsin and related catalytically inactive forms, and to explain a difference titration curve between acetylated δ -chymotrypsin, in which the α -amino group of isoleucine-16 is free to ionize, and fully acetylated δ -chymotrypsin. The

pK_a value 9.7 for the *N*-terminus of elastase is therefore in agreement with the fact that native elastase in solution forms a similar salt-bridge involving homologous residues to that in chymotrypsin.

One notable feature of this group, however, is its relative reactivity of 0.045, which shows that the deprotonated amino group remains buried. This is also apparent from the observation that the *N*-terminal peptide is ten times as reactive as the same group in the native protein. These results give us a quantitative estimate of the degree of 'buriedness' of the protonated *N*-terminal amino group of elastase in solution which can be interpreted in terms of the model shown in Scheme 1.

EH represents the conformation of elastase shown in the crystal structure with protonated valine-16 forming an internal ion pair with aspartic acid-194. E'H represents a hypothetical conformation in which valine-16 has moved from its internal cleft so as to leave the protonated amino group free in solvent, perhaps close to the position which it might occupy in the zymogen (Freer, Kraut, Robertus, Wright & Xuong, 1970). E and E' represent the unprotonated amino groups in these conformations. It is reasonable to assume that acetic anhydride reacts only with the E' form, and if we assume a 'normal' pK_a of 7.9 (Tanford, 1962) for E'H at 10°C, the apparent pK_a of 9.7 for the system gives us an equilibrium constant K' of approx. 0.02 for the 'unfolding' of the protonated conformation. On this basis, the reactivity of the unprotonated amino group (0.044) is 30% of that expected from Fig. 6 (0.145) for a free amino group of pK_a 7.9; so K , the equilibrium constant of the unprotonated 'native' structure, would be about 0.4, and the pK_a for the EH form would be about

Scheme 1. Equilibria of aspartic acid-194 and the *N*-terminus.

10.1. These values are, of course, largely illustrative but give us a picture of the stability of the valine-16-aspartic acid-194 ion-pair.

Garel & Labouesse (1970) have reached very similar conclusions for chymotrypsin from studies of the pH-dependence of the binding of indole to acetylated δ -chymotrypsin, in which the *N*-terminus (isoleucine-16) remains the only amino group unsubstituted. Using the nomenclature of Scheme 1 they assume $pK'_a = 7.8$ at 15°C and find pK_a for the EH form 10.5, $K' = 0.07$ and $K = 30$. Recently J. R. Garel (personal communication) has obtained independent measurements of the conformational equilibria in acetylated δ -chymotrypsin by following the rate of the $EH \rightarrow E'$ transition as a function of pH, measuring small changes in absorption or fluorescence between the two forms of the enzyme. Values of $K = 30$, $K' = 0.02$, $pK_a = 10.5$ and $pK'_a = 7.2$ are thereby indicated. Fersht & Requena (1971) have measured the pK_a of the $E'H$ form by measuring the rate of binding of proflavine to α -chymotrypsin as a function of pH. They find that only the EH form binds the inhibitor and that the pH-dependence of the equilibrium of $EH \rightleftharpoons E'H$ corresponds to pK'_a 8.0 for $E'H$, the form with the protonated amino group free in solution.

Hence, whereas chymotrypsin exists predominantly as the E' form at high pH values in elastase, the 'native' E form predominates even at high pH values. These conclusions are confirmed by the observations of Kaplan, Symonds, Dugas & Whitaker (1970) that there was no change in optical rotation ($[\alpha]_{365}$) of elastase over the pH range in which the amino group becomes deprotonated, whereas $[\alpha]$ changes for chymotrypsin as isoleucine-16 is titrated (Hess *et al.* 1970). Moreover, whereas for chymotrypsin the pH-activity curve parallels the ionization state of isoleucine-16 (Oppenheimer, Labouesse & Hess, 1966; Kaplan, 1971) the present results for elastase show that the pH-activity curves for benzoylalanine methyl ester (Kaplan & Dugas, 1969) and acetylalanylalanylalanine methyl ester (Gertler & Hofmann, 1970) do not parallel the ionization state of valine-16. One must conclude that the valine-16-aspartic acid-194 ion-pair is not essential to maintain the activity of elastase towards small ester substrates.

Above pH 10.5, a large increase appears in the reactivity of the *N*-terminus, which approaches that of valine-16 in the isolated *N*-terminal peptide. Smaller increases appear in the same pH range in the reactivity of lysine-87 and lysine-224. This

suggests that an ionization with $pK_a > 11$ controls a dramatic structural transition (or denaturation) in which all of the groups become more accessible to the reagent. This ionization might involve the 'buried' tyrosine residue at position 234.

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