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AMINO GROUPS

Amino groups of proteins are basic groups (p*K* values near 8 for α-NH_2 and 9.5 for ε-NH_2) and are positively charged except at high pH. Only the uncharged form, that which predominates at pH values higher than their p*K*, is reactive as a nucleophile. Higher pH thus usually enhances their reactivity with most reagents.

Acetic Anhydride. Acetylation with acetic anhydride is probably the most frequently used procedure for the modification of protein amino groups. The preferred method for most work is that first described by Fraenkel-Conrat (1957) as follows:

To the protein solution, preferably but not necessarily of high concentration (2-10%), is added an equal volume of saturated solution of sodium acetate; the solution (or suspension) is cooled in an ice bath and then treated with a total amount of acetic anhydride approximately equal to the weight of protein used, distributed over three to six additions in the course of 1 hour at 0° (e.g., 10 mg of protein in 0.1 ml of H_20, 0.1 ml of sodium acetate, five times 2 µl of acetic anhydride). Dialysis or gel filtration can be used to separate the modified protein from unwanted components of the reaction solution.

The high concentration of sodium acetate serves as a buffer and also helps to direct the selectivity of modification to amino groups (see Section 5-1). Acetylation in the absence of high sodium acetate concentrations is sometimes desirable, in which case the same general procedure can be employed substituting a more desirable buffer or maintaining the pH by periodic addition of alkali. More extensive acetylation of tyrosine residues should be expected under such conditions.

Succinic Anhydride. Succinylation of protein amino groups using succinic anhydride proceeds under mildly alkaline conditions similar to those suitable for acetylation with acetic anhydride (see Section 5-1). Variations of the procedure described by Habeeb et al. (1958) are commonly used, such as the following for the succinylation of staphylococcal enterotoxin B (Chu et al., 1969).

In a typical experiment, 180 mg of enterotoxin B was dissolved in 15 ml of 1.0 *M* carbonate buffer at pH 8.0; 20 mg of solid succinic anhydride was added to the stirred solution every 10 minutes for 70 minutes. The reaction solution was maintained at pH 8.0 with 0.1 *N* NaOH by pH-stat titration. The reaction was stopped at either 1 or 2 hours by dilution to 50 ml with distilled water and then dialyzed. Such treatment resulted in the succinylation of approximately half the total amino groups. The extent of succinylation can be expected to vary with different proteins, and the molar excess of reagent employed (i.e., frequently 10- to 20-fold) should be adjusted to achieve the desired extent of modification. Particularly with small amounts of protein, where a correspondingly small amount of succinic anhydride is used, it is frequently convenient to employ the reagent dissolved in a solution of dry dioxane.

Maleic Anydride. Modification of proteins with maleic anhydride proceeds similarly as in the procedure of Butler et al. (1969) for the preparation of maleylchymotrypsinogen:

Bovine chymotrypsinogen A (20 mg) is dissolved in 2.0 ml of 0.1 *M* soldium pyrophosphate buffer, pH 9.0, and treated at 2°C with 300 μl of 1.0 *M* maleic anhydride in redistilled dioxane. The maleic anhydride solution is added in six additions and the pH of the mixture is maintained at 9.0 by the addition of 0.1 *M* NaOH. When the reaction is complete, the maleyl-chymotrypsinogen is desalted by passing it through a column (40 x 3 cm) of Sephadex G-25 in 0.01 *M* NH_3, and the fractions that contain the protein are pooled to give a solution with a protein concentration of about 0.6 mg/ml. More than 90% of the amino groups are blocked.

For unblocking of maleyl-chymotrypsinogen, the solution of maleyl-chymotrypsinogen is adjusted to pH 3.5 formic acid and aqueous NH_3. The solution is then incubated at 37°C for 30 hours after which the reaction is stopped by addition of alkali to raise the pH above neutrality. More than 90% of the maleyl groups are removed by this procedure.

Reaction with 2-methylmaleic anhydride (citraconic anhydride) proceeds under the same conditions. The deacylation procedure, however, is considerably more rapid (Dixon and Perham, 1968; Gibbons and Perham, 1970). Where mild conditions or more rapid deacylation is desirable the latter reagent appears preferable. These reagents are discussed in Sections 5-1.

Cyanate (Carbamylation). Cyanate reacts with amines at pH 5 and above to give substituted ureas (Sections 5-2). For the modification of protein amino groups, variations of the procedure of Stark et al. (1960) are employed. Reaction rates vary relatively little from pH 5 to 1 unit below the p*K* of the group being modified. A buffer or some other means for controlling the pH of the reaction solution such as a pH-stat should, however, be used to prevent its rise due to hydrolysis of the reagent. Modification typically involves a concentration of sodium or potassium cyanate not greater than 1 *M*, at approximately neutral pH for a time, and a temperature sufficient to give the desired extent of reaction. The preparation of carbamylated rabbit γ-globulin (Chen et al., 1962) is given as a typical example.

Rabbit γ-globulin (20 to 30 mg/ml) in 1 *M* KCNO at 38° in pH 8 borate buffer gave after 4.3 and 9 hours, respectively, 76% and 84% carbamylation of the amino groups. The reactions were stopped and excess reagent removed by dialysis.

O-Methylisourea (Guanidination). O-Methylisourea reacts with ε-amino groups of proteins converting lysine residues into homoarginine residues (see Section 5-3). The reaction is strongly pH-dependent, giving greater modification at higher pH. The procedure of Klee and Richards (1957) for the guanidination of pancreatic ribonuclease follows:

The pH of a solution of O-methylisourea hydrochloride is adjusted with 2.5 *M* NaOH to the desired value between 8.5 and 11 as measured at room temperature. This solution is cooled to 2° and added to the sample of ribonuclease to give a final concentration of 0.5 *M* O-methylisourea and 0.5% protein. The reagent is a good buffer in the pH region 9 to 11, and only very small changes in pH are expected from solution of the protein in the adjusted reagent or from the subsequent reaction.

The reaction is allowed to proceed at 2° for periods of time from a few hours to 3 or 4 days. Samples are taken at appropriate intervals and placed in sufficient acetate buffer, pH 5.0, to bring the resulting pH below 7. The extent of reaction obtained at the various pH values and times is shown in Figure 5-8. A pH of 10 or greater is required for extensive reaction.

Ethyl Acetimidate (Amidination). Amidination of proteins with ethyl acetimidate occurs in aqueous solutions between pH 7 and 10. The reaction is strongly pH dependent, being more rapid at higher pH. A procedure similar to that of Wofsy and Singer (1963) for bovine serum albumin is applicable to many proteins.

To achieve 62 to 65% of amidination, 0.14 g ethyl acetimidate hydrochloride and 0.1 ml of 5 *M* NaOH are mixed and added to 5 ml of an approximately 1% protein solution in borate buffer, pH 8.5, $\mu = 0.1$. (The reagent is about 0.2 *M* in a total volume of 6 ml. The amount of 5 *M* NaOH mixed with the reagent prior to addition of the protein solution is somewhat less than that required for neutralization; the preliminary mixing avoids subjecting the protein to extremes of pH.) The pH is adjusted and maintained at 8.3 to 8.6 for 2 hours, the reaction solution being stirred and kept at 0°. After 2 hours, the solution is dialyzed to stop the reaction.

To attain 85% amidination, the same procedure can be used but with an increase in reagent concentration to 1 M. For exhaustive amidination, the combined effect of more reagent, higher pH and temperature, and longer reaction time can be used (Wofsy and Singer, 1963). Amidination is discussed in Section 5-3.

Reductive Alkylation. Reduction of the Schiff bases resulting from the reaction of various aldehydes and ketones with protein amino groups can be used to produce a large variety of substituted proteins (see Section 6-8). The procedure, known as reductive alkylation, can be done between pH 8 and 10 at low temperature (Means and Feeney, 1968).

Reductive methylation of most proteins is best done at 0° in 0.20 *M* borate buffer (pH 9.0). For each milliliter of solution containing 2.5 to 10.0 mg of protein, approximately 0.5 mg of sodium borohydride is added, followed by five increments (0.5 μl/ml of reaction solution) of 37% aqueous formaldehyde solution over a period of 30 minutes. Such treatment generally results in the methylation of more than 80% of the amino groups. The relative amounts of formaldehyde and sodium borohydride indicated are such that a very slight excess of borohydride should remain at the end of the reaction. This is important in that it prevents other possible reactions between the protein and formaldehyde. More extensive modification is best obtained by a repetition of the same procedure. Reductive ethylation using acetáldehyde in place of the formaldehyde solution can be done similarly, but for equivalent treatment gives less extensively modified proteins.

Reductive isopropylations can be done at 0° in 0.18 *M* borate buffer (pH 9.0) containing 10% v/v acetone. Several small portions of sodium borohydride are added (0.5 to 1 mg/ml of reaction solution) to each milliliter of solution containing 2.5 to 10.0 mg of protein, until the desired extent of modification is achieved. The samples can be dialyzed or subjected to gel permeation chromatography to separate the protein from unreacted reagents and reaction products.

Trinitrobenzenesulfonic Acid. The reaction of trinitrobenzenesulfonic acid (TNBS) with protein amino groups takes place at pH values near 7 or above, and can be used to study the effect of amino-group substitution and to quantitatively determine amino groups (Section 6-5). The recommended procedure is essentially that first described by Habeev (1966):

To 1 ml of protein solution (0.6 to 1 mg/ml) is added 1 ml of 4% $NaHCO_3$ (pH 8.5) and 1 ml of 0.1% TNBS in water. The solution is then incubated at 37°-40°C in the dark for a time sufficient to give the desired extent of reaction. Two hours at 40° gives near-quantitative reaction. Lower temperatures give slower and more selective reaction. To quantitate the extent of reaction, 1 ml of 10% sodium dodecyl sulfate should be added to solubilize the protein upon addition of 0.5 ml of 1 *M* HCl. The absorbance of the solution at 335 to 345 mμ is read against a blank containing 1 ml of water instead of protein solution. An extinction coefficient of $1.4 \times 10^4\ M^{-1}\ cm^{-1}$ can be used to calculate the number of amino groups present.

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