COUNTING INTEGRAL NUMBERS OF AMINO GROUPS PER POLYPEPTIDE CHAIN

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Received 11 August 1980

1. Introduction

Proteins have integral numbers of each of the 20 amino acids, but all the currently accepted methods of determining this number measure only a non-integral ratio of moles of amino acid per mole of protein. This value is rarely found to be close to an integer, due to experimental error and uncertainty about the molecular weight of the protein. An alternative method which gives correct integral values was demonstrated recently for cysteine residues [1]; a very similar procedure had been proposed earlier [2,3]. The procedure is independent of any other information about the protein, including its molecular weight.

Here we demonstrate a related procedure for counting the integral number of amino groups, which gives the number of lysine residues plus the α-amino group.

2. Materials and methods

2.1. *Materials*

Bovine pancreatic ribonuclease A (type II-A, Sigma) was purified by CM-cellulose chromatography, as in [4]. Horse ferricytochrome *c* (type VI, Sigma) was used without further purification. Succinic anhydride was purchased from Koch-Light. Urea was the Aristar grade of BDH.

2.2. *Succinylation of proteins*

The proteins were dissolved in water to 1.0 mg/ml, and the pH was adjusted to 7.0 with NaOH. Weighed portions of solid succinic anhydride, varying from initially 10 μg up to 1 mg/ml protein solution, were added with constant stirring. The pH was maintained at 7 by manual addition of 0.04 M, 0.2 M or 0.5 M NaOH. After the pH was constant for ~10 min, a portion of the solution was withdrawn for electrophoretic analysis, and a further portion of succinic anhydride added.

2.3. *Electrophoresis*

Homogeneous polyacrylamide slab gels (14 cm × 16 cm × 1 mm) were prepared by photopolymerizing a solution containing 8 M urea, 11% (w/v) acrylamide, 0.07% *N,N'*-methylenebisacrylamide, 5 μg riboflavin/ml, 0.12% (v/v) *N,N,N',N'*-tetramethylethylene-diamine (pre-adjusted to pH 4 with acetic acid), and 0.05 M Tris–acetate buffer prepared at either pH 3.45 or pH 3.6. 1 M stock solutions of the buffers were prepared by titrating 1 M acetic acid to the given pH with solid Tris. The pH of the final gel solution was not adjusted further; 0.05 M solutions of the buffer were used in the electrode compartments.

The protein solutions obtained after succinylation were mixed with 0.2 vol. glycerol and 0.1 vol. 1 M HCl. Portions of 10–15 μl were layered into 8 mm or 13 mm wide wells of the gel. Electrophoresis was at 250 V (~10 mA/gel) for 2.5–3 h at room temperature. Protein was stained with 0.1% (w/v) Coomassie brilliant blue in 10% (w/v) trichloroacetic acid plus 10% (w/v) sulphosalicylic acid. Destaining was by diffusion in 5% (v/v) methanol plus 7.5% (v/v) acetic acid.

3. Results

The general procedure of determining the number, N, of residues of a given type is to gradually modify them specifically so that a complete spectrum of molecules with 0, 1, 2, ---, N groups modified are present. These species are then counted after separating them by a procedure sensitive to only the number of groups modified. If the modification alters the ionic charge, the separation is readily accomplished by electrophoresis, isoelectric focussing, or ion-exchange chromatography.

Reaction of amino groups with succinic anhydride [5,6] converts them from basic to acidic:

$$-NH_3^+ \xrightarrow{\text{succinic anhydride}} -NH-\overset{\overset{O}{\|}}{C}-CH_2-CH_2-CO_2^-$$

Succinylation of each amino group can then alter the net charge of a protein by up to 2 unit charges.

Progressive succinylation of bovine ribonuclease A and horse cytochrome *c* were monitored by electrophoresis in 8 M urea (fig.1), where the proteins are unfolded. Under these conditions, succinylation of each of the amino groups has an equivalent effect on the electrophoretic mobility. That a complete spectrum of species was generated, with none missing, was ensured by having overlapping spectra between successive samples. The samples illustrated in fig.1 are a selection of the 15–19 total number produced in each case.

Succinylation of ribonuclease A produced 11 additional bands, corresponding to the 10 lysine residues and 1 α-amino group it is known to contain [7]. Horse cytochrome *c* gave 19 additional bands, corresponding to its 19 lysine residues [8]. It has no α-amino group, as there is an acetyl group at the amino terminus of the polypeptide chain.

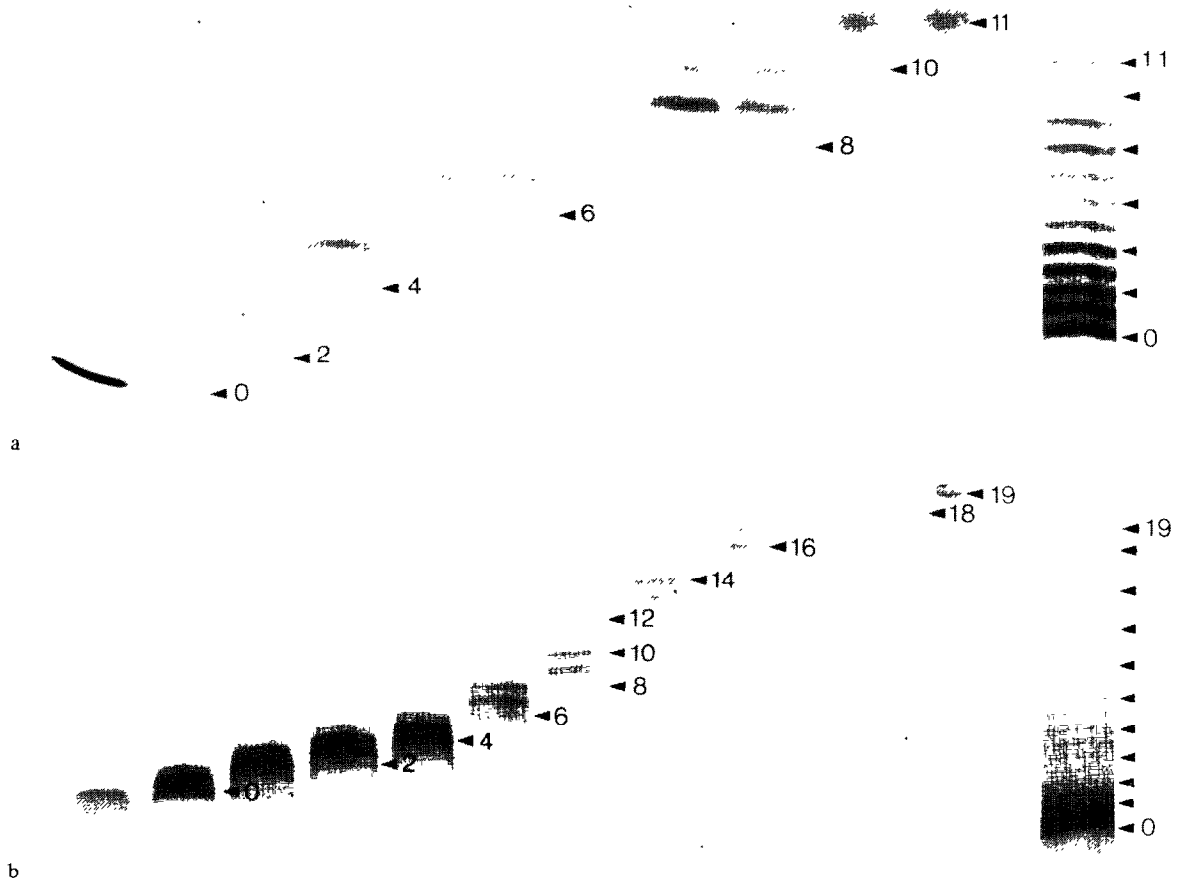

Fig.1. Counting amino groups of (a) bovine ribonuclease A and (b) horse ferricytochrome *c* by electrophoretic resolution of the mixtures produced by progressive succinylation. The various samples illustrated on the left were produced by successive additions of succinic anhydride; the original unmodified protein is on the left and succinylation increases to the right. Electrophoresis was from top to bottom at pH 3.6. The illustrations on the right were of mixtures obtained by combining the individual samples. Electrophoresis was at the lower pH of 3.45, to suppress the ionization of the succinyl groups. Alternative bands are marked by arrows, with the number of succinyl groups indicated for a few of the bands.

4. Discussion

The general method of counting amino acid residues described here for lysine residues and earlier for cysteine residues [1–3] depends upon having a modification reaction which is sufficiently specific and stoichiometric and upon having a separation method able to resolve species differing by only one such modification. Any amino acid for which these two conditions are fulfilled may be counted in this manner.

Succinylation appears to be extremely specific for protein amino groups, as no reaction at other groups was detected. Large amounts of succinic anhydride were added to ensure complete reaction, yet a single electrophoretic band was the limiting product. Reaction with other groups would have been expected to produce at least a unit decrease in net charge, which should have been detectable electrophoretically. Neither of these proteins contains free thiol groups, which are reported to react with succinic anhydride [9]; it may be necessary first to block them.

The $N + 1$ species having $0, 1, 2, \cdots, N$ amino groups succinylated were readily generated by treating the native proteins with small, then increasing, quantities of succinic anhydride. Very small quantities were required initially, so it may be better to carry out the initial reactions at a lower pH, where the amino groups are less reactive [5]. It was not found necessary to unfold the proteins, but this may be prudent with an unknown protein. Whenever the method is used on an unknown protein, a well-characterized protein should be treated in the same way as a control.

Having generated the $N + 1$ species readily, resolving them so that they can be counted is usually the most critical step in the procedure. The method used will depend upon the specific protein. With the two small, basic proteins used here, containing relatively large numbers of lysine residues, the change in net charge produced by complete succinylation was very great, changing them from very basic to very acidic proteins. It was found preferable to suppress the ionization of the succinyl groups by separating the proteins at an acidic pH, where they retained their net positive charge. It was necessary to unfold the proteins so as to make equivalent the succinyl groups on each of the many amino groups of the proteins. Each electrophoretic band contains a large number of isomers, having the same number of succinyl groups but distributed over different amino groups; e.g., succinylation of 10 of the 19 lysine residues of cytochrome *c* could produce 92378 different isomers. It will be noticed in fig.1 that progressive succinylation did not produce a constant change in electrophoretic mobility. Instead, molecules with greater net positive charge had a somewhat smaller electrophoretic mobility per net charge; this probably arises from greater molecular sieving due to expansion of the unfolded proteins because of electrostatic repulsions.

With larger proteins, isoelectric focussing may be preferable to electrophoresis. Progressive carbamylation and acetylation of amino groups of several proteins has been observed to produce discrete bands corresponding approximately to the number of lysine residues [10]. Pure proteins are not required for the general approach described here, so long as all contaminants do not overlap with the spectrum of species generated by succinylation.

Acknowledgements

M. Hollecker was supported by an IAMOV Fellowship from DGRST, France, and by the British Council.

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