Ready separation of proteins from nucleoprotein complexes by reversible modification of lysine residues

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Modification of proteins with citraconic anhydride altered the electrostatic relationship between cationic $\epsilon\text{-NH}_3^+$ groups of lysine residues of proteins and anionic phosphate groups of nucleic acids, thereby destabilizing the nucleoprotein complex. This procedure facilitated the separation of proteins from nucleic acids at pH 4–4.2. The modifying groups were then deacylated from the proteins under acidic conditions (pH 3–6) at 30°C.

The separation and purification of proteins and/or enzymes from nucleoprotein complexes, e.g. ribosomes, is complicated because of interference from nucleic acids. Conventional methods for the separation of proteins from nucleic acids include sodium chloride (Davidson & Waymouth, 1944), guanidinium chloride (Volkin & Cater, 1951), sodium dodecyl sulphate (Kay & Dounce, 1953), phenol, dilute alkali or ethanol. These reagents cause denaturation of the proteins, alter the biological activity and change the conformation of the isolated proteins. In a nucleoprotein complex, proteins and nucleic acids are held together by relatively weak non-covalent forces mainly via electrostatic interactions between cationic $\epsilon\text{-NH}_3^+$ groups of lysine residues of the proteins with the anionic phosphate groups of the nucleic acids. Because of the large number of such weak associations, the complex is quite stable under mild conditions (Spitnik-Elson & Elson, 1976; Lehninger, 1975). Methods for the separation of unaltered proteins from nucleic acids are urgently needed to facilitate purification of intracellular proteins, e.g. enzymes.

By using reversible modification of $\epsilon\text{-NH}_2$ groups of proteins to alter the electrostatic relationship in a nucleoprotein complex, we have developed a simple method for separating proteins from nucleic acids. In the present paper its application to yeast is described.

Experimental

Materials

Brewer's yeast (*Saccharomyces carlsbergensis*) obtained from the Genesee Brewing Co. (New York, NY, U.S.A.) was washed three times with distilled water, freeze-dried and stored at 3–5°C. Citraconic

anhydride and 2,4,6-trinitrobenzenesulphonic acid were purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade or of the highest purity commercially available.

Methods

The Braun Cell Homogenizer (Braun-Metsung, W. Germany) was used to disrupt the yeast cell wall as previously described (Shetty & Kinsella, 1979b). Disrupted yeasts were extracted in water at pH 9.5 (1:20, w/v) and centrifuged at 28 000 *g* for 45 min at 5°C. Citraconic anhydride was added in small increments to the supernatant solution containing proteins and nucleic acids, i.e. nucleoprotein complex. The pH was maintained between 8 and 9 by the addition of 3.5 M-NaOH to neutralize released citraconic anhydride. After stabilization of the pH, portions of the modified protein samples were taken, dialysed against water (pH adjusted to 9.0) for 24 h at 5°C and the extent of modification of amino groups was determined by the trinitrobenzenesulphonic acid method (Hall *et al.*, 1973). The pH of the remaining solution was adjusted to pH 4.2 with 2 M-HCl to precipitate the citraconylated proteins. The protein was recovered after centrifugation, dissolved in water (pH 9.0), dialysed against water (pH adjusted to 9.0) for 24 h at 5°C and freeze-dried. The nucleic acid content of the isolated protein was determined by the orcinol method (Herbert *et al.*, 1971).

Removal of citraconyl groups from citraconylated yeast proteins. The optimum conditions for the removal of citraconyl groups were determined by studying a range of acidic pH values, i.e. pH 3–6, various temperatures in the range 5–50°C and

various incubation times from 1 h to 12 h. In a typical experiment, samples of the citraconylated yeast proteins were dissolved in water (10 mg/ml). The pH of the different samples was adjusted to 3, 4, 5 and 6 with 0.1 M-HCl and held for known intervals of time at 30°C.

The pH of the sample during incubation was controlled by using a Radiometer (Copenhagen, Denmark) TTT60 Titrator. After specified times, the pH of the incubated samples was adjusted to 8.5 with 1 M-NaOH. The samples were then dialysed against water (pH adjusted to 9.0) for 24 h at 5°C to remove the citraconate and then freeze-dried. The extent of deacylation was determined by measuring the available ϵ -NH₂ group of lysine. The effect of temperature on the rate of deacylation was studied at the pH of the most effective deacylation. Nitrogen was determined by the micro-Kjeldahl method (Association of Official Agricultural Chemists, 1975), and protein was determined by the biuret method

as described by Herbert *et al.* (1971), with bovine serum albumin as the standard protein.

Results and discussion

The extent of the decrease in the nucleic acid content of the protein precipitated at pH 4.2 was proportional to the degree of lysine modification, particularly at lower molar ratios of anhydride to protein (Fig. 1). The protein was precipitated at pH 4.2; 85% of the ϵ -NH₂ groups were modified, and it contained 1.86% nucleic acid as contamination (g/100 g of protein) compared with 26% in the unmodified yeast protein isolated under similar conditions. This value is in good agreement with the nucleic acid content of the yeast proteins isolated by the succinylation procedure (Shetty & Kinsella, 1979a,b).

Acylation of proteins with dicarboxylic acid anhydrides increases the net electronegativity in the protein molecules and leads to changes in electrostatic relationships (Means & Feeney, 1971). The addition of two negative charges per lysine ϵ -NH₂ group destabilized the supramolecular nucleoprotein complex by disrupting the non-covalent interactions in the complex. This facilitated the separation of proteins from nucleic acids at pH 4.20. This is consistent with the finding that the separation

Fig. 1. Effect of citraconic anhydride concentration on the extent of ϵ -NH₂-group modification (○) and decrease in nucleic acid content (●) in yeast protein precipitated at pH 4.2

Citraconic anhydride was added in small increments to the pH 9.5 extract containing proteins and nucleic acids from disrupted yeast cells. The pH was maintained between 8 and 9 by the addition of 3.5 M-NaOH. After stabilization of the pH, portions of the modified protein samples were taken, dialysed against water (pH 9.0) for 24 h at 5°C and the amount of lysine modified was determined. The nucleic acid content in the modified protein precipitated at pH 4.20 was also determined.

Fig. 2. Effect of pH on the rate of deacylation of the citraconylated protein at 30°C

The pH of the samples of citraconylated yeast protein (>90% of lysine residues modified) was adjusted to 3.0 (●), 4.0 (○), 5.0 (■) and 6.0 (□) with 0.1 M-HCl and the samples were then incubated for different intervals of time at 30°C. After specified times, the pH of the incubated samples was adjusted to 8.5 with 1 M-NaOH, and they were dialysed against water (pH 9.0) for 24 h at 5°C and freeze-dried. The extent of deacylation was determined by measuring the available ϵ -NH₂ groups of lysine residues.

Scheme 1. Scheme showing separation of proteins from nucleic acids by reversible modification of $\epsilon\text{-NH}_2$ groups in proteins by using citraconic anhydride

of nucleic acids increased with increasing extent of modification of $\epsilon\text{-NH}_2$ groups (Fig. 1). The separation of the proteins from a nucleoprotein complex is similar to the dissociation of an oligomeric protein by modifications with dicarboxylic acid anhydrides in which the subunits are held by non-covalent interactions (Klotz & Keresztes-Nagy, 1962; Hass, 1964; Shetty & Rao, 1978).

Removal of citraconyl groups from the citraconylated protein was performed at acidic pH values. At pH 3.0 and 4.0 complete deacylation of citraconyl groups occurred within 3 h, whereas at pH 5.0 it took 6 h (Fig. 2). However, at pH 6.0 deacylation was slower and levelled off in 6 h at a maximum of approx. 80%. Similar observations were reported by Habeeb & Atassi (1970) for citraconylated lysozyme. Since there was no difference in the rate of removal of citraconyl groups between pH values 3.0 and 4.0 at 30°C, pH 4.0 was selected to study the effect of temperature. The rate of deacylation increased with increasing temperature. At 50°C complete removal of citraconyl groups occurred within 1 h. However, lower temperatures required a prolonged incubation time to achieve complete removal of the modifying groups.

Complete decitraconylation required an acidic pH, which suggested the acid-catalysed hydrolysis of the amide bond. Several mechanisms have been proposed for the reversible deacylation process in which the derivatives were unsaturated cyclic acid anhydrides (Means & Feeney, 1971; Kirby & Lancaster, 1972; Aldersley *et al.*, 1974; Butler & Hartley, 1972; Kirby *et al.*, 1974). Dixon & Perham (1968) showed that both introduction and removal of citraconyl groups from the proteins could be carried out under suitably mild conditions. Results with myoglobin (Singhal & Atassi, 1971) and lysozyme (Habeeb & Atassi, 1970) showed the modification with citraconic anhydride was completely reversible, and the deacylated proteins yielded homogeneous preparations identical with original native proteins with respect to biological properties, conformation and hydrodynamic parameters.

Thus the procedure outlined in Scheme 1 provides a simple method for separating proteins and/or enzymes from nucleoprotein complexes. As reported by Habeeb & Atassi (1970) and Singhal & Atassi (1971), the deacylated proteins are expected to retain their original structure.

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