REACTION OF YEAST INORGANIC PYROPHOSPHATASE WITH MALEIC ANHYDRIDE

S. M. Avaeva, R. U. Beisembaeva, and Z. I. Lebedeva

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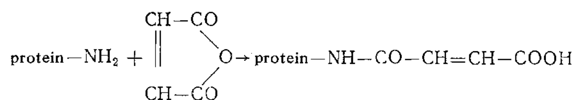
A necessary step in the study of the fundamental structure of proteins is the selective cleavage of a limited number of peptide links. If enzymes are employed for this purpose, especially trypsin, it is very convenient to modify the ε-amino groups of lysine and to hydrolyze only those bonds which are formed with the carboxyl groups of arginine. The ε-amino groups are most often acylated with trifluoroacetic anhydride [1] or succinic anhydride [2–4], and of late the use of maleic anhydride has been proposed for this purpose [5–7].

This paper presents the results of research on the reaction of maleic anhydride with yeast inorganic pyrophosphatase.

The first stages in the isolation of yeast inorganic pyrophosphatase were performed according to Kunitz's procedure [8]. Subsequently the protein was purified on diethylaminoethylcellulose (DEAE-cellulose). However, the protein obtained contained sorbed amino acids. They were removed by dissolving the protein in the minimum quantity of water and then precipitating it with trichloroacetic acid, followed by dialysis against a weak trichloroacetic acid solution and against water. Then the yeast inorganic pyrophosphatase was made to react with maleic anhydride.



The optimum conditions for this reaction were found by analytical experiments. A weighed sample of protein was dissolved in water and treated with a 30- or 60- or 120-fold excess of maleic anhydride at pH 8.5–9.5 and 2° C. The course of the reaction was followed by the decrease in the intensity of the ninhydrin coloration. Table 1 presents the results of the experiments (the intensity of the ninhydrin reaction of the original solution was taken as unity).

Table 1. Reaction of Yeast Inorganic Pyrophosphatase with Maleic Anhydride

Number of experiment	Molar ratio of maleic anhydride to protein	Variation of the relative intensity of the ninhydrin reaction with time, in min				
		0	5	10	15	30
1	30	1	0.39	0.40	0.26	0.23
2	60	1	0.36	0.23	0.20	0.15
3	120	1	0.23	0.24	0.16	0.12
4*	120	1	0.12	—	0.13	0.12

*The protein was previously oxidized with performic acid.

The table shows that with a 120-fold excess of maleic anhydride the reaction is practically completed in 5 min. With a smaller excess of anhydride, the reaction is somewhat slower, but approximately the same degree of acylation of the protein was attained.

The maleic anhydride reaction was also carried out on protein which had been previously oxidized with performic acid (see Table 1, experiment 4). In this case the reaction proceeded still faster and led to the same degree of modification.

In the production of preparative quantities of acylated protein, the reaction was carried for 30 min, using a 1000-fold excess of maleic anhydride. The use of a large excess of maleic anhydride is related to its very great lability [9].

The dinitrophenyl method served to determine the degree to which the protein had been acylated. The dinitrophenylation of the acylated protein was conducted by the usual procedure [10], then hydrolyzed and the quantities of ε-DNP-lysine and lysine determined in an amino acid analyzer. The analysis of the hydrolysate showed the complete absence of ε-DNP-lysine (Table 2, experiment 2). These results prove that, under the conditions described above, quantitative acylation of the ε-amino groups of lysine took place.

Table 2. Determination of the Quantity of ε-DNP-lysine in Hydrolysates of Dinitrophenylated Protein

Number of the experiment	Protein conditions: acylation	Protein conditions: deacylation	Quanity of ε-DNP-lysine, %
1	—	—	91
2	1000-fold excess, 30 min pH 8.5-9.0	—	0
3	1000-fold excess, 30 min pH 8.5-9.0	37°, 24 hr pH 3.5	35
4	1000-fold excess, 30 min pH 8.5-9.0	37°, 24 hr 60°, 10 hr pH 3.5	85
5	1000-fold excess, 30 min pH 8.5-9.0	60°, 16 hr pH 3.5	91

Then it was necessary to find optimum conditions for deacylation. Again the dinitrophenyl method was used to establish the degree of deacylation. The data obtained by dinitrophenylating the original protein served as control. In this connection the protein was dinitrophenylated, then hydrolyzed, and the per cent yield of ε-DNP-lysine determined, taking the sum of ε-DNP-lysine and lysine as 100%; 86% ε-DNP-lysine was found in the hydrolysate. The presence of lysine in the hydrolysate might be due either to incomplete dinitrophenylation of the protein due to its poor solubility, or to the partial decomposition of ε-DNP-lysine during hydrolysis. A series of experiments was undertaken to test this last hypothesis. The DNP-protein obtained was hydrolyzed for 12, 24, and 48 hr with 6 N HCl at 105° C. Only 5% of the ε-DNP-lysine was decomposed after 12 hr, in agreement with literature data [10]. Thus 91% dinitrophenylation of the protein had taken place (Table 2, experiment 1).

To remove the protective groups from the acylated protein it was treated for 24 hr at 37° C with a pyridine-acetate pH 3.5 buffer, again dinitrophenylated, and hydrolyzed. Analysis of the hydrolysate revealed only 35% of ε-DNP-lysine (see Table 2, experiment 3). A supplementary holding of the acylated protein solution for 10 hr at 60° C raised the yield of ε-DNP-lysine to 85% (see Table 2, experiment 4). Treatment of the acylated protein with pyridine-acetate buffer during 16 hr at 60° C and dinitrophenylation led to the formation of 91% of ε-DNP-lysine (see Table 2, experiment 5), i.e., the same quantity as for the original protein. This justified the conclusion that under such conditions deacylation was complete.

The acylated protein becomes much more soluble, which is extremely important for its subsequent hydrolysis by trypsin.

An increased absorption at 250 mμ characterized the UV-spectrum of the acylated protein.

Treatment of yeast inorganic pyrophosphatase with maleic anhydride lowered its sedimentation coefficient from 4.05 to 1.75 S. It has been previously shown that the sedimentation coefficient is also lowered by treating yeast inorganic pyrophosphatase with a large excess of dodecyl sulfate [11]. Our results also show the existence of subunits in the molecule of the enzyme. It should be remarked that dissociation into subunits is observed even when the protein is treated with small quantities (5- or 10-fold excess) of maleic anhydride. The small chemical changes arising from the molecule of yeast inorganic pyrophosphatase after treatment with a small excess of maleic anhydride facilitate the determination of the physicochemical constants of the subunits.

EXPERIMENTAL

1. Yeast inorganic pyrophosphatase. Kunitz's procedure [8] was followed in the first stages of protein separation from stock bakers' yeast (Moscow yeast factory). Plasmolysis of the yeast and fractionation was carried out with ammonium sulfate, followed by autolysis and fractionation by alcohol. The protein obtained after alcohol fractionation was submitted to further purification.

The protein (100 mg) dissolved in 5 ml of water was introduced into a column (3 × 20 cm) filled with DEAE-cellulose. Elution was carried out with a sodium chloride gradient ranging from a 0.01 M tris-HCl solution, pH 7.5,

containing no sodium chloride to a 0.3 M sodium chloride solution in the same buffer. The volume of the mixing vessel was 1 *l*; the rate of elution, 20 ml/hr; and the fraction volume, 5–6 ml. The first 100 ml of eluate was discarded, and the remaining fractions were analyzed for protein content and activity. The fractions that contained active enzyme were combined, lyophilized, dissolved in the minimum quantity of water and dialyzed at 4° C for 20 hr against water, and again dried by lyophilization to yield 40–50 mg of enzyme.

The protein (100 mg) was suspended in 4 ml of water, the insoluble residue removed by centrifuging, and 2 ml of a 30% solution of trichloroacetic acid was added to the solution. After 3 hr the precipitate was separated by centrifuging, suspended in water, and dialyzed at 4° C against a 0.01% solution of trichloroacetic acid for 15 hr and against water for 20 hr, and was then dried by lyophilizing.

2. **Oxidation of pyrophosphatase with performic acid.** Exactly 0.25 ml of 30% hydrogen peroxide was added to 2.5 ml of formic acid. The mixture was kept for 0.5 hr at room temperature in the dark. Then, 2 ml of this solution was added to 20 mg of the protein. After standing for 30 min at room temperature, the reaction mixture was evaporated and the excess of performic acid removed. The yield of oxidized protein was quantitative.

3. **Acylation of the protein with maleic anhydride.**. A) 92 mg of the protein, previously oxidized with performic acid, was dissolved in 5 ml of N NaOH at 0° C, and a suspension of 7.72 g of maleic anhydride in water was added under intensive stirring. The pH of the solution was kept at 8.5–9.0 by adding 2 N NaOH. The reaction mixture was dialyzed against water for 48 hr at 4° C and was then dried by lyophilization. The yield of acylated protein was quantititative.

B) The acylation of analytical quantities of protein (~1 mg) with a 30-, 60-, or 120-fold excess of maleic anhydride was carried out under similar conditions. The course of the reaction was followed by a decrease in the intensity of the ninhydrin coloration. For this purpose samples were taken after 5, 10, 15, and 30 min and transferred to a solution containing 0.5 ml of pH 5.0 acetate buffer and 0.5 ml of the ninhydrin reagent. The mixture was heated for 15 min on a boiling water bath, and then cooled. After the addition of 2 ml of 50% ethanol, the color intensity was measured with an SF-4 spectrophotometer at 570 mμ.

4. **Dinitrophenylation of the protein.** Twelve milligrams of yeast inorganic pyrophosphatase was dinitrophenylated by Sanger's method [10]. The DNP-protein obtained was hydrolyzed for 12, 24, and 48 hr with 6 N HCl at 105° C. The hydrolysates were evaporated, the excess of HCl was removed, and the quantities of ε-DNP-lysine and lysine were determined. The acylated protein (see section 3A) was similarly dinitrophenylated, hydrolyzed for 12 hr, and analyzed.

5. **Removal of protective groups from the acylated protein.** Ten milligrams of protein was dissolved in pyridine-acetate buffer, pH 3.5, and kept either for 24 hr at 37° C, or for 24 hr at 37° C and 10 hr at 60° C, or for 16 hr at 60° C. The protein was then dinitrophenylated, hydrolyzed, and analyzed for its ε-DNP-lysine and lysine contents.

ε-DNP-lysine and lysine were analyzed in a Hitschi amino acid analyzer, with a column (10 × 0.9 cm) filled with spherical resin No. 3105. Table 2 presents the analytical results, as averages of 3–5 experiments.

6. **Sedimentation analysis of the acylated yeast inorganic pyrophosphatase.** Ten milligrams of the acylated protein was dissolved in 1 ml of 0.1 M tris-HCl buffer at pH 8.0. The sedimentation of the protein was studied with a "Spinco E" analytical ultracentrifuge at a velocity of 59,700 rpm.

CONCLUSIONS

The reaction of yeast inorganic pyrophosphatase with an excess of maleic anhydride at 2° C for 5 min leads to the acylation of all ε-amino groups of lysine residues.

The protective groups are completely removed at pH 3.5 for 16 hr at 60° C. Acylation with maleic anhydride is accompanied by the dissociation of the yeast inorganic pyrophosphatase into its subunits.

REFERENCES

1. R. F. Goldberger and C. B. Anfinsen, Biochem., 1, no. 3, 401-405, 1962.
2. C. H. Li, J. S. Dixon, and D. Shung, J. Am. Chem. Soc., 80, 2587, 1958.

3. A. F. S. A. Habeeb, H. G. Cassidy, and S. J. Singer, Biochem. Biophys. Acta, 29, 587, 1958.
4. C. H. Li and J. Bertch, J. Biol. Chem., 235, 2638, 1960.
5. P. I. Butler, J. I. Harris, B. S. Hartley, and R. Leberman, Biochem. J., 103, no. 3, 78, 1967.
6. M. H. Freedman, A. L. Grossberg, and D. Pressman, Biochem., 7, 1941, 1968.
7. J. I. Harris and R. N. Perham, Nature, 219, 1025, 1968.
8. M. Kunitz, Arch. Biochem. Biophys., 92, 270, 1961.
9. A. C. Buton, N. A. Fuller, and S. G. Perry, J. Chem. Soc., no. 5, 2918, 1967.
10. J. Bailey, Methods of Protein Chemistry [Russian translation], 138, 1965.
11. S. M. Avaeva, E. A. Braga, and A. M. Egorov, Biofizika, 1143, 1968.

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Moscow State Univeristy