INHIBITION OF YEAST IN ORGANIC PYROPHOSPHATASE BY THE ESTER OF GLYCINE AND BY HYDROXYLAMINES

N. Yu. Fink, T. I. Nazarova, and S. M. Avaeva

UDC 577.15.02:577.15.04

In a study of the mechanism of the action of yeast inorganic pyrophosphatase, it was found [1] that during the enzymatic reaction a phosphorylated enzyme is formed in which a phosphoric acid residue is covalently bound to one of the functional groups of the active center. On the basis of the results of hydrolysis of the phosphorylated protein under various conditions [2], the hypothesis was put forward that this group is a glutamic or aspartic acid carboxy group. In the present work we discuss how certain reagents interacting with carboxy groups affect the enzymatic activity of inorganic pyrophosphatase.

The reaction with 0.5-1.0 M glycine methyl ester in the presence of water-soluble carbodiimides at pH 4.75 is widely used for the exhaustive modification of carboxy groups in proteins [3]. We used conditions close to the standard conditions. The inorganic pyrophosphatase was treated with 0.5 M glycine methyl ester and 0.05 M N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate (CMEC) at pH 5.0 and 30°C. The number of freecarboxy groups in the inorganic pyrophosphatase was not known accurately, but the protein molecule contained about 100 aspartic acid and glutamic acid residues [4]. Under the optimum condition, the reaction took place rapidly, and the protein molecule, undergoing far-reaching changes, lost its enzymatic activity, as was to be expected (Fig. 1a).

Since inorganic pyrophosphatase is extremely rapidly denatured at pH 5.0, the reaction was performed in the presence of inorganic pyrophosphate, which considerably stabilizes the enzyme. This made it possible to follow the course of the modification with time.

When only 0.05 M CMEC was used, the enzymatic activity fell rapidly (see Fig. 1b). As is well known, carbodiimides can react with carboxy groups giving derivatives of O-acylureas which, apparently, was also observed in the present case. Inorganic pyrophosphatase is extremely sensitive to carbodiimide even at a concentration of CMEC of $5 \cdot 10^{-4}$ M, i.e., two orders of magnitude lower than in the experiment described above; in 30 minutes the enzymatic activity had fallen by 15%.

However, at pH 5.0 irreversible inhibition of the enzyme took place even without CMEC, when the reaction mixture contained only glycine methyl ester (see Fig. 1c). This phenomenon is possibly due to the presence in the molecule of inorganic pyrophosphatase of, at it were, a special form of activated carboxy group which does not need additional activation by carbodiimide for the reaction with glycine methyl ester. The presence of such an activated carboxy group in the protein would also enable us to explain the formation of a macroergic acyl phosphate bond which probably takes place in the phosphorylation of the enzyme by inorganic phosphate [5].

Calcium pyrophosphate (0.001 M), which is a competitive inhibitor of inorganic pyrosphosphatase, protects the enzyme from the influence of glycine methyl ester to a considerable extent (see Fig. 1d). This shows that the action of the glycine methyl ester is directed to one of the groups of the active center, since calcium pyrophosphate is bound to the same section of the enzyme as the substrate – magnesium pyrophosphate.

Compounds containing activated carboxy groups, such as esters or acyl phosphates, readily react with hydroxylamine forming hydroxamic acids [6]. Consequently, we then determined how hydroxylamine affects the properties of the inorganic pyrophosphatase, above all in the process of phosphorylating the enzyme with inorganic phosphate. For this purpose we performed two parallel experiments in which phosphoryla-

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 235-240, March-April, 1975. Original article submitted October 30, 1973.

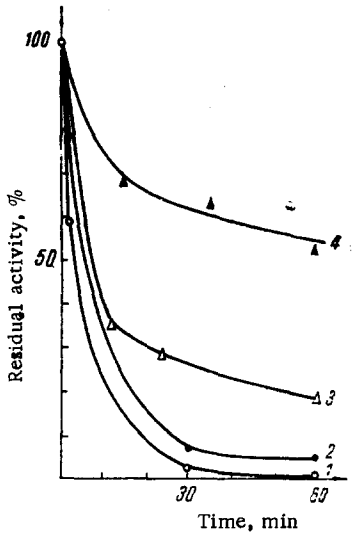

Fig. 1. Inhibition of the enzyme by the methyl ester of glycine and by N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate, pH 5.0, 30°C: 1) 0.5 M glycine methyl ester, 0.05 M CMEC, 0.001 M $Na_4P_2O_7$; 2) 0.05 M CMEC, 0.001 M $Na_4P_2O_7$; 3) 0.5 M glycine methyl ester; 4) 0.5 M glycine methyl ester, 0.001 M calcium pyrophosphate.

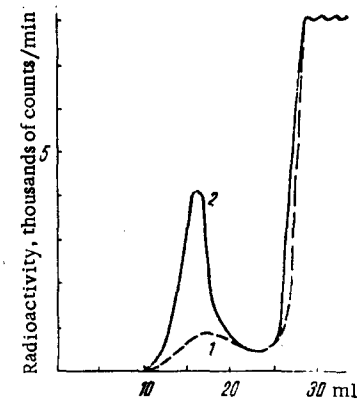

Fig. 2. Isolation of phosphorylated inorganic pyrophosphatase by gel filtration on Sephadex G-50: 1) experiment with 1 M NH_2OH; 2) experiment with 1 M NaCl.

tion by radioactive inorganic phosphate was performed at pH 5.0 by a method developed previously [5]. In one experiment, the reaction mixture contained 1 M hydroxylamine and in the other 1 M sodium chloride. After the isolation of the phosphorylated protein it was found that in the experiment with hydroxylamine the inclusion of the phosphorus label in the protein amounted to only 31% of its inclusion in the parallel experiments (Fig. 2). This is in harmony with the hypothesis that hydroxylamine interacts with the group of the active center that binds the phosphate residue in the formation of a phosphorylated protein.

In subsequent experiments we found how hydroxylamine affects the enzymatic activity of inorganic pyrophosphatase. Figure 3 shows the dependence of the activity on the concentration of the agent at pH 7.0. As can be seen from the graph, at a concentration of hydroxylamine of 0.01-0.2 M some activation of the enzyme is observed, and then the activity rapidly falls and even at a concentration of hydroxylamine of 0.15 M it is only 50% of the activity in a control experiment. Under these conditions, the depth of inhibition depends on the time of incubation of the enzyme with the hydroxylamine, and when the reaction mixture is diluted the enzymatic activity is completely restored. Although this contradicts the theoretical scheme of the reaction (the formation of hydroxamic acid should be irreversible), nevertheless this result is not unexpected.

It has been shown previously that at high concentrations (0.8-2.0 M) hydroxylamine reversibly suppresses the activity of a number of ATPases [7], for which the presence of carboxy groups in the active centers has also been suggested. It may be assumed that in inorganic pyrophosphatase and some other enzymes a special mechanism exists which constantly "liberates" the carboxy group of the active center and the phosphate residue or inhibitor molecule binding with it. Such a role may be played by, for example, one of the functional groups of the protein.

The behavior of the enzyme in the presence of the colored N-(2-hydroxy-5-nitrobenzyl)hydroxylamine (HNBH) at pH 7.0 has also been considered. For this reagent we found the same characteristics as for hydroxylamine (reversibility of the inhibition and independence from the time). However, this reagent had a considerable effect at far lower concentrations (Fig. 4). Thus, 50% suppression of activity was achieved even at a concentration of the HNBH of $1.6 \cdot 10^{-4}$ M, i.e., three orders of magnitude lower than for the unsubstituted hydroxylamine (see Fig. 3). The results of a study of the inhibiting action of HNBH at different pH values have shown that its maximum effectiveness is precisely at pH 7.0 (Fig. 5) – the pH optimum of the action of inorganic pyrophosphatase.

With the simultaneous action on the enzyme of hydroxylamine and CMEC, a gradual irreversible loss of enzymatic activity took place which amounted to 55% after an hour (Fig. 6, curve 1). The reaction was performed under mild conditions at pH 7.0 and 30°C. Hydroxylamine in a concentration of 0.01 M not only did not inhibit the enzyme but even somewhat activated it (see Fig. 3), and at a concentration of CMEC of $4 \cdot 10^{-10}$ M the activity had fallen by only 15-20% (see Fig. 6, curve 2). But the addition to the reaction mixture of 0.001 M pyrophosphate led to the complete protection of the enzyme from the action of the hydroxylamine while scarcely decreasing the degree of inactivation due to the presence of CMEC itself (see Fig. 6, curves 3 and 4). The action of hydroxylamine is apparently directed almost completely to a group of the active center, while the carbodiimide modifies the accessible carboxy groups nonspecifically.

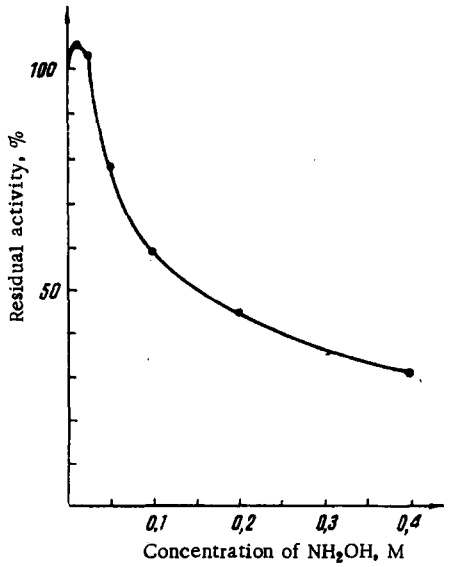

Fig. 3. Dependence of the activity of the enzyme on the concentration of NH_2OH, pH 7.0, 30°C.

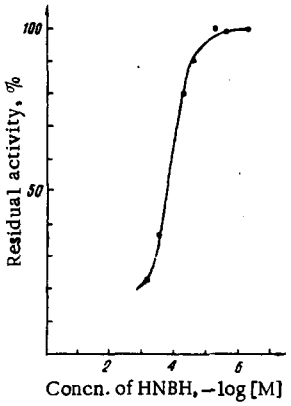

Fig. 4

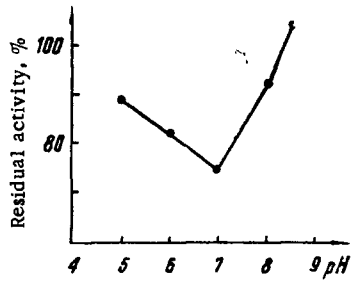

Fig. 5

Fig. 4. Dependence of the activity of the enzyme on the concentration of N-(2-hydroxy-5-nitrobenzyl)hydroxylamine, pH 7.0, 30°C.

Fig. 5. Dependence of the degree of inhibition of the enzyme by N-(2-hydroxy-5-nitrobenzyl)hydroxylamine on the pH, $2.0 \cdot 10^{-5}$ M HNBH, 30°C.

In a study of the reaction described above at other pH values, it was found that at pH 6.5 inhibition is accelerated and takes place to the extent of 70% in an hour. However, with a further decrease in the pH a rapid denaturation of the protein begins. The addition of inorganic pyrophosphate to the reaction mixture to stabilize the enzyme leads to its protection from inhibition.

The effect observed in the combined action of hydroxylamine and CMEC is possible due to a Lossen rearrangement. D. E. Koshland et al. [8] have established that hydroxamic acids undergo this rearrangement under the influence of water-soluble carbodiimides at pH 5.0 and room temperature. The inhibition of the enzyme by HNBH in the presence of CMEC remains reversible, which is in harmony with the proposed explanation, since an unsubstituted nitrogen atom in a hydroxylamine is necessary for the rearrangement. In the course of the rearrangement, the hydroxamic acid residue formed by the reaction of the hydroxylamine with the carboxy group of the enzyme is converted into an amino group. However, aspartic and glutamic acid residues are converted, respectively, into diaminopropionic and diaminobutyric acid residues.

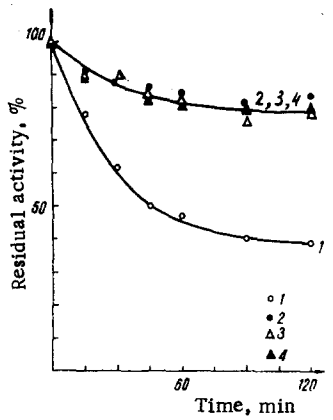

Fig. 6. Reaction of the enzyme with hydroxylamine and N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate, pH 7.0, 30°C; 1) 0.01 M NH_2OH, $4.3 \cdot 10^{-4}$ M CMEC; 2) $4.3 \cdot 10^{-4}$ M CMEC; 3) 0.01 M NH_2OH, $4.3 \cdot 10^{-4}$ M CMEC, 0.001 M $Na_4P_2O_7$; 4) $4.3 \cdot 10^{-4}$ M CMEC, 0.001 M $Na_4P_2O_7$.

If in the inhibition of inorganic pyrophosphatase by hydroxylamine and CMEC the process described above does actually take place, the possibility arises for the subsequent acquisition of a direct proof of the presence of a carboxy group in the active center of the enzyme.

EXPERIMENTAL METHOD

Inorganic pyrophosphatase was isolated from yeast by the method of Braga and Avaeva [9]. The concentration of protein was determined spectrophotometrically at 280 mμ on the basis that protein in a concentration of 1 mg/ml has an absorption of 1.45 optical density units. The molecular weight was taken as 70,000 [10]. Preparations of the enzyme with specific activities of 650 and 970 units, expressed in μmoles of inorganic phosphate liberated by 1 mg of protein per minute at 30°C, were used. The incubation mixture for determining activities contained 1 ml of 0.01 M of $Na_4P_2O_7$, 1 ml of 0.01 M $MgSO_4$, and 4 ml of 0.1 M acetate–ammonia buffer, pH 7.0. The time of incubation with the enzyme was 10 minutes. The amount of phosphate formed was found by a modification of Fiske's method [11]. The CMEC, synthesized in the Institute of Organic Chemistry of the Siberian Branch of the Academy of Sciences of the USSR, was kindly given to us by V. M. Stepanov. The HNBH was synthesized from 2-hydroxy-5-nitrobenzyl chloride [12] and acetoxime by a method similar to that of Exner [13]. The concentration of HNBH was determined spectrophotometrically at 410 mμ, taking the molar absorption as $1.7 \cdot 10^4$ at alkaline pH values [14]. Grade ch. d. a. ["pure for analysis"] $HCl \cdot NH_2OH$ was used after two recrystallizations from ethanol. In the preparation of the solutions, the pH was brought to the required value with 1 M NaOH. All the inhibition experiments were performed at 30°C.

Inhibition of Inorganic Pyrophosphatase by Glycine Methyl Ester. The experiments were performed in 0.03 M veronal–acetate buffers, pH 5.0 and 7.0. The concentration of the enzyme was $2 \cdot 10^{-7}$ M. of the hydrochloride of glycine methyl ester 0.5 M, of CMEC 0.05 or 0.0005 M, and of $CaCl_2$ and $Na_4P_2O_7$ 0.001 M. The volume of the reaction mixture was 1 ml. To determine the enzymatic activity, after predetermined intervals of time 15- to 25-μl samples were taken. The residual activity was referred to the activity in control experiments.

Reaction of the Enzyme with Radioactive Inorganic Phosphate in the Presence of NH_2OH. The reaction was performed in 0.2 M acetate buffer, pH 5.0, at room temperature. In a volume of 0.4 ml, the reaction mixture included 0.21 mg of enzyme, $1.25 \cdot 10^{-3}$ M Na_2HPO_4 containing ^{32}P, and 1.0 M NH_2OH, pH 5.0, or 1.0 M NaCl. After 3 h, a solution of sodium dodecyl sulfate was added to give a concentration of 0.1%. After 20 h, the reaction mixture was deposited on a column (1.5 × 25 cm) of Sephadex G-50 and was eluted with 0.05 M tris-HCl buffer, pH 7.2. Fractions with a volume of 1.5 ml were collected, and the radioactivities were counted in a Nuclear-Chicago scintillation counter (USA).

The inclusion of ^{32}P amounted to 0.39 mole of phosphate/mole of protein in the experiment with NaCl and 0.12 in the experiment with NH_2OH.

Inhibition of the Inorganic Pyrophosphatase by NH_2OH and HNBH. The reaction mixture with a volume of 5 ml contained NH_2OH or HNBH of the given concentration, $2 \cdot 10^{-3}$ M $Na_4P_2O_7$, and 0.1 M acetate buffer, pH 7.0. Either immediately or after predetermined intervals of time, 1 ml of 0.01 M $MgSO_4$ was added to it and the activity of the enzyme was determined. In a study of the irreversibility of inhibition, 15- to 25-μl samples were taken from the reaction mixture and added to a solution for the determination of activity. In control experiments the reaction mixtures contained, in place of NH_2OH, NaCl of the corresponding concentration.

To investigate their dependence on the pH, the experiments were performed in 0.03 M veronal–acetate buffers, pH 5.0-8.5. The concentration of HNBH was $2 \cdot 10^{-5}$ M and of $Na_4P_2O_7$ $2 \cdot 10^{-3}$ M. The activity was determined in the reaction mixture by adding $MgSO_4$ and was referred to the activity in a control experiment without HNBH measured at the same pH.

Reaction of the Enzyme with NH_2OH and HNBH in the Presence of CMEC. In a volume of 5 ml, the reaction mixture contained 0.01 M NH_2OH or $2 \cdot 10^{-3}$ M HNBH, $4.3 \cdot 10^{-4}$ M CMEC, the protein in a concentration of $0.7 \cdot 10^{-7}$ M, and 0.1 M acetate buffer with pH 7.0 or 0.03 M veronal–acetate buffer with pH 5.0-6.5, and also, where shown, $2 \cdot 10^{-3}$ M $Na_4P_2O_7$. After predetermined intervals of time, samples were taken for activity measurements.

SUMMARY

1. Glycine methyl ester is an inhibitor of yeast inorganic pyrophosphatase at pH 5.0 both in the presence and in the absence of N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate (CMEC). Calcium pyrophosphateprotects the enzyme from inhibition.

2. Hydroxylamine and N-(2-hydroxy-5-nitrobenzyl)hydroxylamine are reversible inhibitors of inorganic pyrophosphatase.

3. In the presence of CMEC hydroxylamine is an irreversible inhibitor of the enzyme. Inorganic pyrosphosphate protects the enzyme from inactivation.

4. The results obtained confirm the hypothesis that there is an activated carboxy group in the active center of yeast inorganic pyrophosphatase.

LITERATURE CITED

1. S. M. Avaeva and A. T. Mevkh, Biokhimiya, 34, 1088 (1969).
2. S. M. Avaeva and T. I. Nazarova, Khim. Prirodn. Soedin., 243 (1970); T. I. Nazarova, G. A. Bektenova, and S. M. Avaeva, in: Collection of Papers at a Scientific Conference of the Interfaculty Laboratory of Bioorganic Chemistry of Moscow State University [in Russian], Moscow (1971), p. 215; T. I. Nazarova, N. Yu. Fink, and S. M. Avaeva, FEBS Letters, 20, 167 (1972).
3. D. G. Hoare and D. E. Koshland, J. Amer. Chem. Soc., 88, 2057 (1966).
4. R. Eifler, R. Glaesmer, and S. Rapoport, Acta Biol. Med. German, 17, 716 (1966).
5. T. I. Nazarova and S. M. Avaeva, Biokhimiya, 38, 169 (1973).
6. F. Lipmann and L. Tuttle, J. Biol. Chem., 159, 21 (1945).
7. C. F. Chignell and E. Titus, Biochim. Biophys. Acta, 159, 345 (1968); M. Makinose, Eur. J. Biochem., 10, 74 (1969); A. Martonosi, J. Biol. Chem., 244, 613 (1969); G. Inesi, E. Maring, A. J. Murphy, and B. McFarland, Arch. Biochem. Biophys., 138, 285 (1970).
8. D. G. Hoare, A. Olson, and D. E. Koshland, J. Amer. Chem. Soc., 90, 1638 (1968).
9. É. A. Braga and S. M. Avaeva, Biokhimiya, 38, 344 (1973).
10. J. W. Ridlington, Y. Yang, and L. G. Butler, Arch. Biochem. Biophys., 153, 714 (1972).
11. H. Weil-Malherbe and H. Green, Biochem. J., 49, 286 (1951).
12. Org. Syntheses [Russian translation], Moscow, Coll. 3 (1952), p. 478.
13. O. Exner, Chem. Listy, 50, 779 (1956).
14. T. Yamamoto, A. Yoda, and Y. Tonomura, J. Biochem., 69, 807 (1971).