INFLUENCE OF SH REAGENTS ON THE ACTIVITY OF INORGANIC YEAST PYROPHOSPHATASE

S. M. Avaeva and O. V. Koval'chuk

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The present paper gives the results of a study of the action of SH reagents on the activity of inorganic pyrophosphatase: p-chloromercuribenzoate, 5,5'-dithiobis(2-nitrobenzoic acid), iodoacetate, iodoacetamide, cystine and cysteine. The reactions of the enzyme with these compounds were performed in tris-HCl buffer, pH 7.2, at room temperature. After predetermined intervals of time, the enzymatic activity was measured and compared with the activity of a control preparation kept under the same conditions without the SH reagent.

The action on the enzyme of a tenfold molar excess of p-chloromercuribenzoate did not lead to any change in activity with respect to protein during a day. An increase in the concentration of the reagent to $3 \cdot 10^{-5}$ M (one hundred-fold molar excess) slightly affected the enzymatic activity: after reaction for 12 h, the residual activity amounted to 81%. Only when a 10^{-3} M solution of p-chloromercuribenzoate (5000-fold excess of the reagent) was used did rapid inactivation begin to take place: after 30 min, the residual activity was 30%, but this figure scarcely changed during 24 h.

Table 1 shows the results of the experiments with the above-mentioned reagents. It can be seen from this table that 5,5'-dithiobis(2-nitrobenzoic acid), just like p-chloromercuribenzoate, scarcely changed the activity of the enzyme. Fairly high concentrations of iodoacetic acid and iodoacetamide also had no influence on the activity of inorganic pyrophosphatase under the given conditions. The slow inactivation of the enzyme in the presence of large amounts of iodoacetamide is probably the result of the denaturation of the protein under the action of this reagent. The decrease in the activity of the enzyme on treatment with cystine and cysteine is apparently not connected with a modification of the group of the active center, since the inactivation process was slow and did not go to completion; the residual activity amounted to not less than 50% after reaction for 12 h.

TABLE 1

SH reagent	Final conc., M	Ratio of reagent to enzyme	Time	Residual activity, %
p-Chloromercuribenzoate	$3 \cdot 10^{-5}$	100	10 min	95
			12 h	81
	$1{,}5 \cdot 10^{-3}$	5000	10 min	30
			24 h	26
5,5'-Dithiobis(2-nitrobenzoic acid)	$1 \cdot 10^{-5}$	100	10 min	95
			1 h	95
			12 h	80
Iodoacetate	$1 \cdot 10^{-2}$	30 000	10 min	96
			24 h	96
Iodoacetamide	$8 \cdot 10^{-3}$	30 000	10 min	95
			12 h	78
	$3 \cdot 10^{-2}$	100 000	10 min	100
			3 h	45
			24 h	8
Cysteine	$1 \cdot 10^{-3}$	3000	10 min	95
			1 h	95
			12 h	50
Cystine	$1 \cdot 10^{-3}$	3000	10 min	75
			24 h	75

The experimental material permits the statement that the sulfhydryl groups of inorganic yeast pyrophosphatase are not involved in the active center of the enzyme.

The results of our investigations contradict those of other authors [1-3]. Bailey and Webb [1] have suggested, on the basis of the fact that the activity of the enzyme decreases after incubation with $5 \cdot 10^{-3}$ M iodoacetate at 38°C for 15 min, that inorganic yeast pyrophosphatase is a SH enzyme. Since the modification of other groups as well, may take place under such conditions, this conclusion cannot be regarded as suf-

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ficiently convincing. Rapoport et al. [2, 3], on the basis of the reaction of the enzyme with p-chloromercuribenzoate, 5,5'-dithiobis(2-nitrobenzoic acid), and N-acetylsulfamoylphenylmaleimide concluded that a sulfhydryl group participates in the active center: the action of one mole of N-acetylsulfamoylphenylmaleimide selectively blocked one SH group, the activity of the enzyme falling by 90% after 48 h. However, in the light of what has been said above, one cannot but take into account the possibility of a disturbance of the native conformation of the protein on the introduction of such a voluminous substituent as N-acetylsulfamoylphenylmaleimide.

Of the inorganic pyrophosphatases isolated from other sources, that from E. coli has been studied in most detail [4]. It was established that the free SH groups of this enzyme are necessary for the formation of its quaternary structure. It has been shown that p-chloromercuribenzoate, iodoacetate, and iodoacetamide are not inhibitors for the inorganic pyrophosphatase of the vegatative cells and spores of Bacillus subtilis [5].

EXPERIMENTAL

The p-chloromercuribenzoate was a Chemapol preparation thrice reprecipitated with 1 N HCl from solution in 1 N NaOH, washed with water, and dried in a vacuum desiccator over P_2O_5.

The 5,5'-dithiobis(2-nitrobenzoic acid) was synthesized by Ellman's method [6]. After two recrystallizations from glacial acetic acid, its mp was 236-238°C.

The iodoacetic acid was purified by recrystallization from hexane.

The iodoacetamide was recrystallized from hot water.

The L-cystine hydrochloride and L-cysteine hydrochloride were Reanal preparations and were not subjected to further purification.

The inorganic pyrophosphatase was isolated from bakers' yeast by Kunitz's method [7]. The preparation obtained after the first recrystallization had a specific activity of 36 units. The modification reaction was performed by adding 9-20 μl of a concentrated solution of the reagent to a $3 \cdot 10^{-7}$ M solution of the enzyme in 0.01 M tris-HCl buffer, pH 7.2, at 23-25°C. The final concentrations of the reagents are shown in Table 1. Before mixing, all the solutions were brought to pH 7.2.

SUMMARY

The retention of the activity of the enzyme under conditions for the modification of the sulfhydryl groups by various SH reagents shows that the SH groups are not involved in the active center of inorganic yeast pyrophosphatase.

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