SULFHYDRYL GROUPS OF YEAST INORGANIC PYROPHOSPHATASE AND THEIR RELATIONSHIP TO THE ACTIVITY OF THE ENZYME

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

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Information is given in many publications on the investigation of the state of the cysteine residues in yeast inorganic pyrophosphatase, but questions relating to the disulfide bonds, the number of free SH groups and their role in the manifestation of the activity of the enzyme have not so far been answered. Eifler et al. [1], have found three hemicystine residues in an acid hydrolyzate of the protein (mol. wt. 60,000); two of them form an S–S bond, and there is one free SH group which is essential for activity [2]. Negi and Irie [3, 4], have shown that in the presence of denaturing agents all free SH groups are modified by sulfhydryl reagents without preliminary reduction, which indicates the absence of S–S bonds in the protein. However, in Butler's opinion [5], the protein does contain an S–S bond which is important for activity. Hansen et al. [6] have established that the enzyme has two free SH groups which react with 5,5'-dithiodi-2-nitrobenzoic acid (DTNB) in the presence of 8 M urea; S–S bonds are absent, since after reduction the number of reactive SH groups does not change.

The present paper gives new experimental results which enable the proceeding results to be generalized and the existing contradictions to be explained.

The study of the reaction of inorganic pyrophosphatase with p-chloromercuribenzoate and DTNB have shown that no modification of the SH group takes place in the absence of denaturing agents. When a SH-reagent and urea or sodium dodecyl sulfate are added simultaneously to the protein, the rate of the reaction is again very low.

For the prolonged treatment of the protein, a suitable SH reagent is iodoacetic acid, which forms a stable compound with cysteine – carboxymethylcysteine (CM-cysteine) which may then be isolated from an acid hydrolyzate of the modified protein. For the present work we used iodoacetic acid labelled with ^{14}C and the same acid in the nonradioactive form.

To answer the question of the existence of S–S bonds, experiments were performed to modify the enzyme with labelled iodoacetic acid in the absence and in the presence of a reducing agent. In experiment *a*, [^{14}C]iodoacetic acid in a denaturing buffer was added to the enzyme and the reaction was performed as described above in the absence of a reducing agent. In experiment b, the protein was previously reduced with dithiothreitol, and then the labelled iodoacetic acid was added to it. In experiment c, nonradioactive iodoacetic acid was added to the protein, and then, successively, dithiotheitol and [^{14}C]iodoacetic acid. In all the experiments, the method of denaturation was the same.

Below we give information on the carboxymethylation of inorganic pyrophosphatase in the presence and in the absence of dithiothreitol (pH 8.6, 10^{-3} M EDTA, concentration of the enzyme $5 \cdot 10^{-6}$ M, concentration of [^{14}C]iodoacetic acid 0.01 M).

Sequence of addition of the reagents to the protein	No. of [^{14}C]-CM-cysteine residues per mole of protein
[^{14}C]iodoacetic acid (a)	3.4
Dithiothreitol; [^{14}C]iodoacetic acid (b)	3.7
Iodoacetic acid, dithiothreitol [^{14}C]iodoacetic acid (c)	0.25

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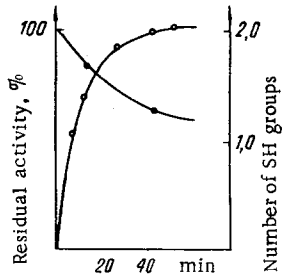

Fig. 1. Change in the activity of the pyrophosphatase on the modification of the SH groups by DTNB (pH 8.3, 0.01 M EDTA), 25°C, concentrations of enzyme and inhibitor, respectively, $5 \cdot 10^{-6}$ and $5 \cdot 10^{-5}$ M.

As can be seen from a comparison of experiments *a* and b, the number of residues of CM-cysteine did not increase after the reduction of the enzyme with dithiothreitol. The absence of [^{14}C]-CM-cysteine under the conditions of experiment c also shows that no new SH groups appear after the reduction of the enzyme. The results obtained show that there are no disulfide bonds in inorganic pyrophosphatase and that the question of the number of cysteine residues in the protein can be discussed. From the large number of experiments performed in the manner of experiment *a* using iodoacetic acid preparations with different specific radioactivities it can be seen that the number of CM-cysteine residues formed per mole of protein averages 3.12, while when the percentage decomposition of the CM-cysteine during acid hydrolysis is taken into account [7] it amounts to 3.5. Since the modified protein was not separated from the unchanged protein, this value is somewhat low, and the total number of free SH groups in yeast inorganic pyrophosphatase must be considered to be not less than four. The results we have obtained, and those given in the literature, show the steric inaccessibility of the SH groups. Another reason for the lack of the activity of the SH groups of the native enzyme may be their participation in complex formation with metal atoms strongly bound to the enzyme [8].

The complexone ethylenediaminetetraacetic acid (EDTA) causes the inactivation of the enzyme, its rate increasing with a rise in temperature and a fall in the concentration of the protein. At 20-25°C in the presence of 10^{-2} M EDTA the enzyme retains its activity for several hours, and at 37°C using $5 \cdot 10^{-6}$ M, $4 \cdot 10^{-6}$ M, and $-1 \cdot 10^{-6}$ M of protein its residual activity after 1 h amounted to 66%, 60%, and 30-40%, respectively.

However, it is important that in the presence of reagents causing modification of SH groups the rate of inactivation of the enzyme is considerably increased. The residual activity in the presence of 10^{-2} M EDTA with $5 \cdot 10^{-6}$ M p-chloromercuribenzoate is 16% (60%),* and with $1.7 \cdot 10$†M iodoacetic acid and iodoacetamide it is 20% (95%) and 40% (95%), respectively. The concentration of enzyme was $0.5 \cdot 10^{-6}$ M, pH 8.3, 37°C, 1 h.

In order to increase the specificity of the inactivation process, the reaction with SH reagents in the presence of 10^{-2} M EDTA was performed at 25°C for 20-24 h at pH 8.3. Then DTNB was added and the degree of modification was determined spectrophotometrically, using $\varepsilon_M^{412} = 13{,}600$. These experiments showed that at 25°C, just as at 37°C, reaction of the SH groups leads to a decrease in reactive of the enzyme (Fig. 1).

The inactivation of the enzyme in the presence of a tenfold excess of DTNB is kinetically of the pseudo-first order with a rate constant of $1 \cdot 10^{-2}$ min^{-1}. After the addition of the DTNB, a reaction with the SH groups of the proteins begins, and the modification reaction has pseudo-first order at a rate constant of $7 \cdot 10^{-2}$ min^{-1}. The increase in absorption at 412 nm in 45-60 min corresponds to the modification of 2 SH groups. At this time the activity of the enzyme amounts to 60% of the initial activity. During a day, the activity falls to zero. In this case, modification is performed not with DTNB but with iodoacetic acid.

The protein was kept in a buffer containing 10^{-2} M EDTA for 12-15 h, and then iodoacetate was added and the reaction mixture was incubated at 37°C for 3 h. The reaction of the iodoacetate with the protein denatured with 8 M urea and 1% sodium dodecyl sulfate, but in the absence of EDTA, was performed in parallel. After the addition of the EDTA to the protein, one to two reactive SH groups appeared. The denaturation of the enzyme to which no EDTA had been added caused the appearance of 2.5 SH groups. However, as stated above, the total number of SH groups reacting with iodoacetate in the presence of both EDTA and denaturing agents is 3.5. The results obtained permitted the conclusion that the SH groups of inorganic pyrophosphatase can be separated into two classes. The first class includes groups showing reactivity only in the presence of EDTA. Their number is apparently two. The other groups react only after the far-reaching degradation of the protein successively with 8 M urea and 1% sodium dodecyl sulfate. Their amount can be determined from the difference between the total number of SH groups and the number of SH groups liberated in the presence of EDTA.

* The figures obtained in the absence of EDTA are given in parentheses.

† As in Russian original – Publisher.

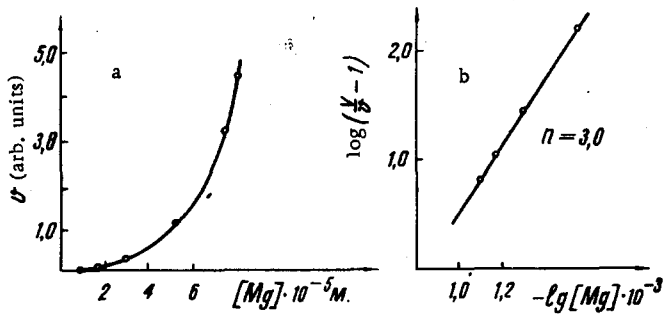

Fig. 2. Influence of free concentrations of Mg^{2+} on the activity of inorganic pyrophosphatase modified with DTNB at a free concentration of pyrophosphate of 0.015 M (pH 5.6, 25°C) (*a*) and the results "A" expressed in Hill's coordinates (b).

It has been shown previously that the enzyme molecule contains four to six metal atoms [8]. The maximum value of Hill's coefficient for Mg^{2+} found from the dependence of the rate of hydrolysis of pyrophosphate on the concentration of the free form of Mg^{2+} is 2.7 [9]. We determined Hill's coefficient for the enzyme in which two sulfhydryl groups had been modified by DTNB (Fig. 2). Hill's coefficient did not change after the modification of the enzyme with DTNB. Thus, modification of the SH groups in the presence of EDTA does not cause a change in the number of centers of binding of metal atoms participating in the formation of the active complex. This conclusion is in harmony with the results obtained previously: the inactivation of the enzyme caused by the reaction of the SH groups in the presence of EDTA takes place considerably more slowly than modification. The available experimental results do not make it possible accurately to determine the function of the SH groups, but there is no doubt that in the presence of EDTA the SH groups of the enzyme become reactive and their modification leads to the inactivation of the enzyme. One of the reasons for this is, possibly, a change in the active structure of the enzyme when the SH groups are modified, which leads to considerable acceleration of inactivation under the action of EDTA.

On the basis of the results obtained, we have attempted to generalize literature information on the number of SH groups and their role in the activity of the enzyme. The total number of hemicystine residues, namely three, was determined earlier by the oxidation of the protein with performic acid [1, 3, 4] or by reaction with DTNB and p-chloromercuribenzoate in the presence of a reducing agent [1, 4], while the molecular weight of the protein was taken as 60,000. At the presnt time, it has been established that the molecular weight of yeast inorganic pyrophosphatase is 70,000 [6]. In this case, the number of hemicystine residues become 3.5. The results given by Hansen et al. [6], showing the determination of only two SH groups in the reaction of the enzyme with DTNB in the presence of 10^{-3} M EDTA remain unclear. Nevertheless, the time of contact of the enzyme with a denaturing buffer was too brief to show all the reactive SH groups.

On the basis of literature sources it is impossible to draw any conclusions relating to the role of the SH groups in the enzymatic activity of inorganic pyrophosphatase, since in some cases the modification of the SH groups was performed in the presence of denaturing agents [3, 4, 6] and in others only indirect information on the influence of SH reagents on the activity of the enzyme with no determination of the degree of modification of the SH groups was obtained [1, 2, 10].

EXPERIMENTAL

Purification of the Reagents. The iodoacetic acid was purified by repeated recrystallization from CCl_4, mp 80-82°C. The [2-^{14}C]iodoacetic acid was a product of the Radiochemical Centre, Amersham, with a specific radioactivity of 34 mCi/mole. Before the beginning of the experiment, this material was diluted by the addition to 0.75 μmole of [2-^{14}C]iodoacetic acid of 34-43 moles of nonradioactive iodoacetic acid neutralized with NaOH. The p-chloromercuribenzoate was a Chemapol preparation which was reprecipitated three times with 1 N HCl from solution in 1 N NaOH and, after being washed with water, was dried in a vacuum desiccator over P_2O_5. The 5,5'-dithiodi-2-nitrobenzoic acid, a preparation of the firm Calbiochem, was used without additional purification. The urea and the tris were purified by recrystallization from 60% ethanol.

The inorganic pyrophosphatase was isolated from mother baker's yeast by Kunitz's method [11]. The amino-acid composition of the electrophoretically homogeneous protein corresponded to literature data [1, 3].

The enzymatic activity was determined from the rate of cleavage of sodium pyrophosphate to orthophosphate in 0.2 M ammonium acetate buffer, pH 7.0, at 30°C at concentrations of pyrophosphate and Mg^{2+} of 0.01 and 0.03 M, respectively. Inorganic phosphate was determined by the method of Weil-Malherbe and Green [12] or by using a semiautomatic inorganic phosphate analyzer [9]. The activity of the enzyme was 900-1200 units.

Synthesis of [^{14}C]Carboxymethylcysteine. Cysteine hydrochloride (~ 10 mg) was dissolved in 0.3 ml of water, and tris was added to pH 8-9. Nitrogen was passed for 30 min in order to eliminate oxygen from the solution, and then 0.03-0.05 ml (3-4 μmole) of dilute [^{14}C]iodoacetic acid was added and the mixture was left at room temperature for 2-3 days. Then an equal volume of 12 N HCl was added and hydrolysis was performed at 105°C in vacuum for 22 h. After the usual working up, the hydrolyzate was deposited on the column of an amino-acid analyzer. The fractions were collected from the column and the specific radioactivity of the caroxymethylcysteine was determined, its amount being calculated from the ninhydrin peak and by measuring the total reactivity in the CM-cysteine peak.

Reaction of Inorganic Pyrophosphatase with [^{14}C]Iodoacetic Acid. A. Without Preliminary Reduction. Nitrogen was passed through 0.2-0.4 ml of a buffer solution, pH 8.6, containing 0.1 M tris-HCl 8 M urea, 10^{-3} M EDTA and 10^{-2} M [^{14}C]iodoacetic acid for 30 min. Then the enzyme was added ($1 \cdot 10^{-6}$ to $5 \cdot 10^{-6}$ M) and the reaction mixture was left in the dark at room temperature. After 24-26 h, sodium dodecyl sulfate was added to a concentration of 1.0-1.5% and the reaction mixture was incubated at 37°C for 3-4 h. Then the protein was separated from the low-molecular-weight products by gel filtration in a column (1 × 30 cm) of Sephadex G-25 equilibrated with 0.005 M tris-HCl buffer having pH 8.6. The fractions containing protein were treated with an equal volume of 12 N HCl and hydrolyzed in vacuum at 105°C for 22 h.

B. After Reduction with Dithiothreitol. Nitrogen was passed for 30 min through a buffer solution, pH 8.6, containing 0.1 M tris-HCl, 8 M urea, and 10^{-3} M EDTA, and then the protein was added ($5 \cdot 10^{-6}$ M) and the reaction mixture was left at room temperature in the dark. After 24-36 H, sodium dodecyl sulfate was added to a concentration of 1.0-1.5% and the mixture was incubated at 37°C for 1 h. Then dithiothreitol was added ($3 \cdot 10^{-3}$ M) and the mixture was incubated further at 37°C for two h, after which [^{14}C]iodoacetic acid was added to a concentration of 0.02 M and the reaction mixture was kept at room temperature for 3 h. The modified protein was separated by gel filtration on Sephadex G-25, and the protein fraction was hydrolyzed as described in experiment A.

C. After Preliminary Treatment of the Protein with Unlabeled Iodoacetic Acid. The protein ($5 \cdot 10^{-6}$ M) was treated with nonradioactive iodoacetic acid ($2 \cdot 10^{-4}$ M) in 0.1 M tris-HCl buffer, pH 8.6, with 10^{-3} M EDTA, for 36 h at room temperature, and then sodium dodecyl sulfate was added and the mixture was incubated at 37°C for another 1 h. The subsequent treatment was the same as for experiment B.

Determination of [^{14}C]-CM-Cysteine in a Hydrolyzate of the Labeled Protein. A hydrolyzate of the protein modified with [^{14}C]iodoacetic acid was evaporated to dryness, dissolved in citrate buffer, pH 2.2, and deposited on the 50-cm column of a Beckman Unichrom automatic amino-acid analyzer. Simultaneously, 0.03-0.05 μmole of nonradioactive CM-cysteine was deposited on the column as a marker. Analysis was performed in the usual way, and after being mixed with ninhydrin, the elutate was collected for analysis for radioactivity: fractions with a volume of 1.8-2.0 ml were taken by means of a collector and the radioactivity of each fraction was measured. The fractions containing the CM-cysteine were combined and the amount of [^{14}C]-CM-cysteine was determined as the ratio of the total radioactivity in the CM-cysteine peak to the specific radioactivity of the initial [^{14}C]-iodoacetic acid.

Determination of the Radioactivity of Compounds Containing ^{14}C. To 0.05-0.50 ml of the solution were added 10 ml of dioxane scintillator (60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of diphenyloxazolylbenzene, 200 ml of ethanol, and dioxane to a total volume of 1 liter) and counting was performed in a Mark II counter (USA) for 4-8 min.

Modification of the SH Groups of Inorganic Pyrophosphatase with DTNB. The enzyme ($6 \cdot 10^{-6}$ M) was kept at room temperature for 12 h in 0.1 M tris-HCl buffer, pH 8.3, containing 10^{-2} M EDTA, and then DTNB was added ($4 \cdot 10^{-4}$ M). The modified protein was separated from the excess of reagent after 3-4 h by gel filtration on Sephadex G-25, the protein being eluted with water.

Determination of the Enzymatic Activity of the Inorganic Pyrophosphatase Modified with DTNB in the Presence of Varying Concentrations of Mg^{2+}. The enzymatic reaction with the modified enzyme was carried out at 25°C in 0.025 M morpholinoethane sulfone buffer, pH 5.6, at a constant free concentration of pyrophosphate of 0.015 M with variation in the concentration of the forms of Mg^{2+} from 0.08 to 0.8 mM. The

initial concentrations of pyrophosphate and Mg^{2+} were calculated by using the stability complex of the magnesium pyrophosphate complex which is 1950 M^{-1} [9]. The enzymatic reaction was begun by adding the enzyme to the solution of Mg^{2+} and pyrophosphate in buffer (total volume 2 ml) and was stopped after 5 min by the addition of 0.15 ml of 6 N H_2SO_4. The amount of phosphate formed was determined on a semiautomatic phosphate analyzer [9].

CONCLUSIONS

1. The total number of sulfhydryl groups in yeast inorganic pyrophosphatase has been determined.

2. The absence of disulfide bonds in the molecule of the protein has been shown by the reaction with [^{14}C]iodoacetic acid before and after the reduction of the enzyme with dithiothreitol.

3. It has been shown that the modification of two of the SH groups of inorganic pyrophosphatase in the presence of EDTA is accompanied by the inactivation of the enzyme.

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