REVERSIBLE ACID DISSOCIATION OF THERMOSTABLE INORGANIC PYROPHOSPHATASE FROM *BACILLUS STEAROTHERMOPHILUS*

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Received 31 October 1979

1. Introduction

During the last years extensive investigations of reversible dissociation of multisubunit enzymes were accomplished in connection with the question whether or not catalytic activity of oligomeric enzymes is an intrinsic property of the folded monomers. According to these results it seems in general to require association of the monomers to obtain the fully active oligomeric enzyme (e.g., dehydrogenases [1–5] or aldolase [6]).

Continuing our work on the thermostable PPase from *B. stearothermophilus* [7,8] this paper deals with the acid dissociation of hexameric PPase into the inactive trimers and monomers. Complete reassociation and reactivation could be achieved by readjustment to the original pH value. Studies on heat stability were accomplished for the trimeric and hexameric state of PPase.

2. Materials and methods

The PPase was prepared from *B. stearothermophilus* strain ATCC 12980 as in [7]. A homogeneous enzyme preparation with spec. act. 180 IU/mg was used throughout the experiments.

The enzyme activity was assayed as in [7]. In residual activity determinations the reactivation in the activity assay was negligible during the time of measurement (0.25 μg protein/ml). The protein concentration (c) was measured photometrically using an $A^{0.1\%}_{280\,nm}$ of 1.10 [7].

Inactivation/dissociation were achieved by dilution of the native PPase (stock solution: 1 mg/ml 0.1 M Tris–HCl (pH 7.5)) with 0.1 M Tris–acetate buffer (pH 5.4) or 0.1 M glycine buffer (Sörensen) (pH 2.6) for 2–15 h at 4°C.

The determinations of the pH-dependent equilibrium between hexameric and trimeric state were performed in 0.1 M Tris–acetate buffer at 20°C.

Reactivation/reassociation by readjustment to the original pH value were carried out by dilution of the inactivated enzyme with 0.1 M Tris–HCl buffer (pH 7.5) (reactivation buffer).

The heat inactivation at pH 5.4 and 7.5 was performed at 70°C and 80°C. After pre-inactivation at pH 5.4 the PPase activity was assayed after reactivation.

The molecular weights of PPase at different pH values were estimated by density gradient centrifugation with a preparative ultracentrifuge model L 2-65 B (Beckmann) in a linear sucrose gradient of 5–20% (pH 7.5, 5.4, 2.6), and by gel filtration on a column of Sephadex G-200 (1.5 × 95 cm, pH 7.5, 5.4). Chymotrypsinogen A (Serva/Heidelberg), bovine serum albumin (Mann Res. Lab., New York), lactate dehydrogenase (VEB Arzneimittelwerk Dresden) and γ-globulin (Schwarz-Mann, Orangeburg, NY) served as marker proteins.

SDS–PAGE was performed as in [9], except that 10–20% linear gradient slab-gels were used. Human serum albumin (VEB Serum- und Impfstoffwerk Dessau), alcohol dehydrogenase (VEB Arzneimittelwerk Dresden) and cytochrome c (Serva/Heidelberg) were used as the standards. Gels were stained with Coomassie brillant blue G-250.

Abbreviations: PPase, inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Fluorescence measurements were performed with a fluorescence spectrophotometer, model Hitachi MPF-2A (λ_{exc} = 295 nm, c = 25–100 μg/ml).

3. Results and discussion

3.1. *Effect of acid pH on native structure and enzymatic activity*

It was reported that the thermostable PPase from *B. stearothermophilus* was mol. wt 122 000 as determined by ultracentrifugal analysis [10] or 140 000 ± 15 000 as estimated by density gradient centrifugation [7]. Here the enzyme was confirmed to be mol. wt 120 000 by Sephadex G-200 gel filtration in Tris–HCl buffer (pH 7.5). The PPase subunit was determined by SDS–PAGE to be mol. wt 20 000. From this it follows that the enzyme consists of 6 subunits. For the thermostable PPase from *B. stearothermophilus* strain NCA 2184 2 subunits with mol. wt 70 000 were ascertained [10]. The reason for this discrepancy remains unclear. It is of interest to note that the molecular weight of the thermostable *Escherichia coli* PPase and their subunits are identical with that of our enzyme [11]. For the subunits of thermostable PPase from *Thiobacillus thiooxidans* a mol. wt 20 000 was also determined but in this case the tetramer is reported to represent the native enzyme [12].

The quarternary structure of PPase from *B. stearothermophilus* strain is strongly dependent on the pH value. Complete dissociation into inactive subunits could be achieved by shifting the pH to 2.6. Inactive trimeric state of PPase arises in over pH 6.4–5.2 where hexamers and trimers are in equilibrium. At pH 5.0 the enzyme exists mainly in the trimeric state. The monomeric and trimeric states of the enzyme were detected by density gradient centrifugation at the appropriate pH values, the trimeric state was additionally confirmed by Sephadex G-200 gel filtration at pH 5.4. The pH-dependent inactivation of PPase is illustrated in fig.1. Mg^{2+} did not significantly influence the state of equilibrium.

The dissociation into the trimeric- or monomeric state caused no change of the maximum position of the native protein fluorescence (λ_{em} = 329 nm). In a control experiment with Gu–HCl denatured PPase there was a red shift of the fluorescence emission up to 350 nm. Therefore, no denaturation accompanies the dissociation of PPase.

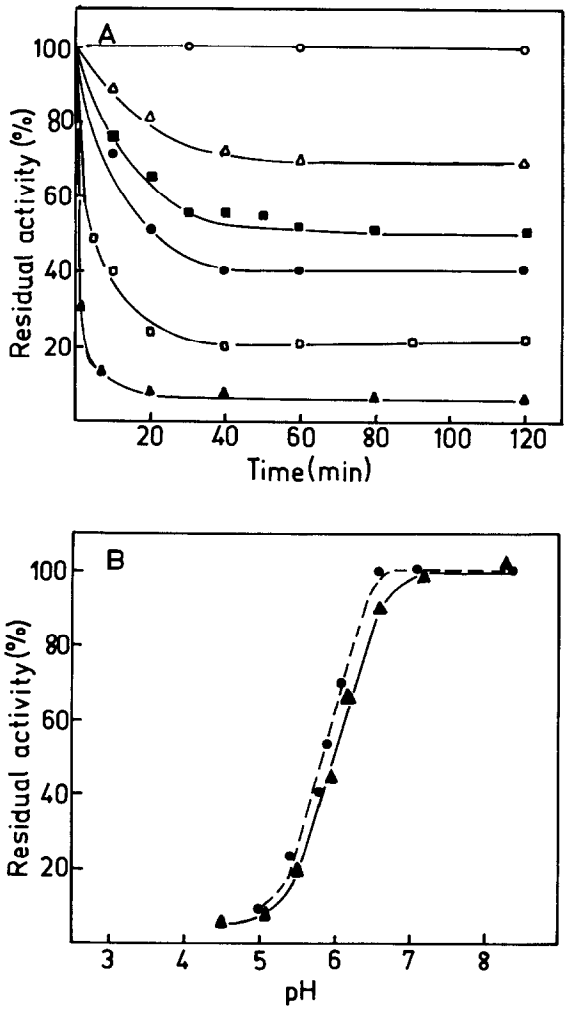

Fig.1. Influence of pH value on the inactivation of PPase in 0.1 M Tris–acetate buffer (20°C) c = 25 μg/ml. (A) Kinetics of inactivation at various pH values: 6.5–8.4 (○); 6.1 (△); 5.9 (■); 5.7 (●); 5.4 (□); 5.0 (▲). The time course of inactivation follows a first-order kinetics. (B) pH dependence of residual activity after reaching the equilibrium (60 min); without Mg^{2+}, taken from fig.1A (●); Mg^{2+} = 4 × 10^{-3} M (▲).

3.2. *Reactivation*

The pH-dependent dissociation and inactivation of PPase is a fully reversible process. Reactivation was started by dilution of aliquots of previously inactivated enzyme solution with reactivation buffer (see section 2) followed by measuring the activity after different time intervals. In order to slow down the reactivation rate the reactivation experiments were carried out at 15°C. The kinetics of reactivation is shown in fig.2.

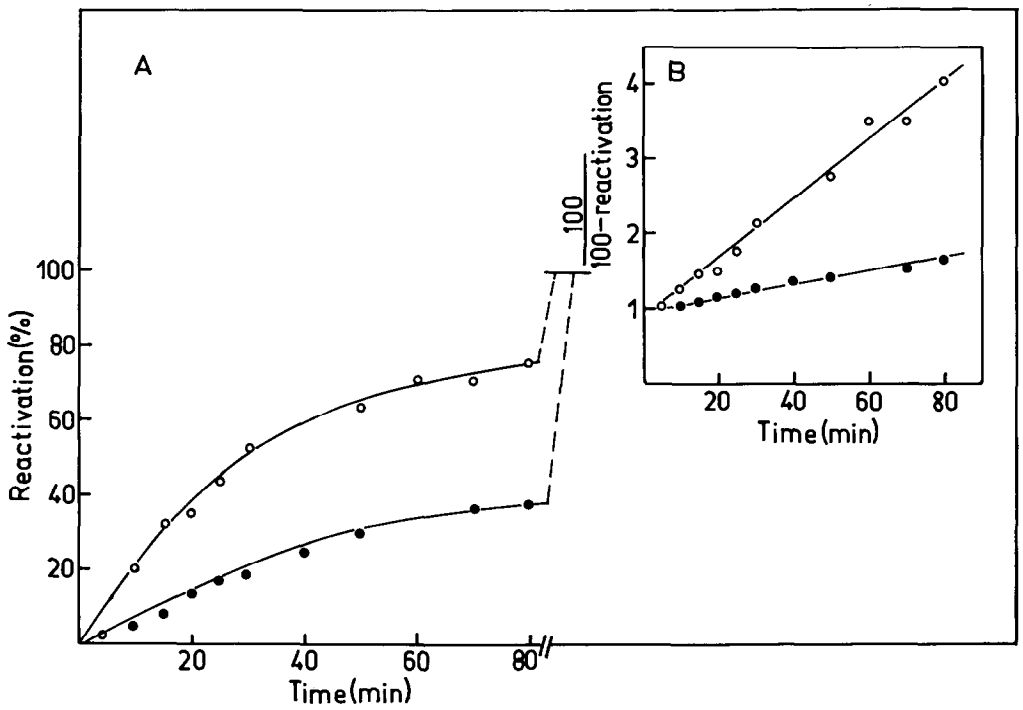

Fig.2. Kinetics of reactivation of PPase after inactivation at pH 5.4 (0.1 M Tris–acetate buffer) for 15 h at 4°C. (A) Reactivation in 0.1 M Tris–HCl buffer (pH 7.5) at 15°C. c = 40 μg/ml (○); c = 10 μg/ml (●). (B) Determination of second-order rate constant k ($mM^{-1}.s^{-1}$) from data given in (A). k = 2.67 (○); k = 2.23 (●).

Under the indicated experimental conditions the reactivation follows a second-order reaction (fig.2B). The reactivated enzyme turns out to be indistinguishable from the native PPase with respect to molecular weight, fluorescence emission spectrum, heat resistance, and enzymatic activity. Within the experimental error the reactivation results in ~100% at the enzyme concentration tested (10–40 μg/ml). The reactivation rate is decreased at decreasing protein concentration as to be expected from a second-order reaction.

3.3. *Thermostability of the hexameric- and trimeric state*

Recent studies demonstrated the high thermostability of native PPase from *B. stearothermophilus* [7,10]. In this respect the heat resistance is dependent on the presence of metal ions. For example, in the presence of 4×10^{-3} M Mg^{2+}, ~90% of the initial activity is maintained after 30 min at 80°C and pH 7.5, but only 50% in the absence of Mg^{2+} (not reversible). In connection with our results on acid dissociation of PPase it was of interest to know about thermostability of the trimeric state of PPase. Therefore, heat incubations have been carried out in the presence of 4×10^{-3} M Mg^{2+} at both 70°C and 80°C at pH 5.4 (c = 200 μg/ml). Under these conditions there exist ~25% hexamer and 75% trimer. After heat incubations the enzyme activities were measured after reactivation at pH 7.5 (c = 40 μg/ml). A residual activity of 100% was found for 30 min incubation at 70°C, and ~60% for the same incubation time at 80°C. From this it follows that also the trimeric state exhibits a considerable thermostability in the presence of Mg^{2+}. Because of the relatively rapid equilibrium between monomeric and trimeric as well as the trimeric and the hexameric state it is not possible to compare the degree of thermostability of different states with the experimental conditions used*.

* After completion of this manuscript a paper was published [13] describing an association/dissociation behaviour of the same enzyme depending on Mg^{2+} or the anions present in the buffer solution. Furthermore, the subunit was corrected in this paper to be mol. wt 18 000 instead of 70 000 described in [10]

4. Conclusions

Thermostable inorganic pyrophosphatase from *B. stearothermophilus* represents an active hexamer at pH 7.5. The enzyme is rapidly dissociated into inactive monomers with mol. wt 20 000 by shifting the pH to 2.6. Over pH 6.4–5.2 an equilibrium exists between hexamer and inactive trimer. The dissociation is not accompanied by denaturation. Complete reassociation and reactivation to hexameric native PPase could be achieved by readjustment of the pH to 7.5. The catalytic activity is restricted to the hexameric state of PPase but in contrast the thermostability is not.

Acknowledgements

The author thanks Mrs H. Wessner for performing the density gradient centrifugation and Dr W. E. Höhne and Dr C. Frömmel for helpful discussions.

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