Cold lability of the mutant forms of *Escherichia coli* inorganic pyrophosphatase

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Abstract The variants of *Escherichia coli* pyrophosphatase carrying the substitutions Glu20→Asp, His136→Gln or His140→Gln are inactivated, in contrast to the wild-type enzyme, at temperatures below 25°C: their activity measured at 25°C decreases with decreasing the temperature of the stock enzyme solution. The inactivation is completely reversible and is explained by cold-induced dissociation of these hexameric enzymes into less active trimers.

Key words: Inorganic pyrophosphatase; Cold inactivation; Site-directed mutagenesis; Quaternary structure; Sedimentation

1. Introduction

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) catalyzes reversible transfer of the phosphoryl group from pyrophosphate to water, the reaction that provides a thermodynamic pull for such vital biosynthetic processes as protein and nucleic acid syntheses [1]. PPase is a convenient model system of the phosphoryl transfer enzymes, which is one of the largest classes of enzymes; yet their mechanisms of action are still not fully understood [2,3].

PPase of *Escherichia coli* is homohexameric [4] and contains 175 amino acid residues per subunit [5]. Its three-dimensional structure has been determined at a 2.5–2.7 Å resolution [6,7]. The monomeric unit is exceedingly irregular and belongs to the $\alpha+\beta$ class of folds. The hexamer is arranged as an almost perfect octahedron, in which two trimers interact via the α-helices containing His-136 and His-140 [6]. The *ppa* gene encoding *E. coli* PPase has been cloned and sequenced [5], and a number of site-directed mutants of this enzyme have been prepared and partially characterized [3,8]. While being essential for identification of catalytic groups, these studies also helped to identify several active site residues (Glu-98, Lys-104, Tyr-55 and Tyr-141) that are important for structural integrity [9,10].

In the course of these studies, we found that, while being stable at elevated temperatures, some mutants of *E. coli* PPase, in contrast with WT-PPase, lose activity on incubation at 0°C. In this report, we show that such cold inactivation is caused by destabilization of quaternary structure.

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Abbreviations: PPase, inorganic pyrophosphatase; PP_i, pyrophosphate; WT-PPase, *E. coli* wild-type PPase.

2. Materials and methods

The missense mutations in the *E. coli* PPase gene were performed by the site-specific mutagenesis method of Kunkel et al. [11] using a single mutagenic primer at a time as described by Lahti et al. [10]. To confirm the mutations and to ensure that no secondary mutations had arisen, the whole *ppa* gene was sequenced [12] using a universal primer and four *ppa*-specific primers designed for this purpose [9]. The wild-type and mutant PPases were produced and purified to homogeneity as described by Lahti et al. [9].

Initial rates of PP_i hydrolysis were estimated from continuous recordings of phosphate liberation obtained with an automatic phosphate analyzer [13]. The reaction mixture of 5–20 ml total volume contained 23 μM PP_i, 20 mM $MgCl_2$, 50 μM ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetate (EGTA) and 0.1 M Tris-HCl (pH 7.2). Reactions were started by adding enzyme and were carried out at 25°C. The reaction progress curves were strictly linear for at least 3 min in all cases. One unit of enzyme activity is defined as the amount of the enzyme hydrolyzing 1 μmol PP_i per minute.

Analytical ultracentrifugation was carried out in a Spinco E instrument equipped with an optical system for making scans at 280 nm. Sedimentation velocity was measured at 60,000 rpm, and the sedimentation coefficient, $s_{20,w}$, was calculated using a standard procedure [14]. Sedimentation equilibrium runs were performed at 20,000 rpm for 24 h, and the molecular mass was calculated according to Chernyak et al. [15]. The partial specific volume of PPase (0.72 cm^3/g) was calculated from its amino acid composition [5].

Enzyme incubations were carried out in 0.15 M Tris-HCl (pH 7.2) or 0.1 M HEPES-KOH (pH 7.2) containing 50 μM EGTA and either 0.5 mM dithiothreitol (E20D-PPase) or 1 mg/ml bovine serum albumin (H136Q- and H140Q-PPases) for enzyme stability. For ultracentrifugation, albumin was replaced by 0.5 mM dithiothreitol. Unless otherwise noted, the pH values cited refer to the temperatures at which particular experiments were conducted.

3. Results

Most studies described below were carried out with the *E. coli* PPase variant in which Glu-20 was replaced by Asp (E20D- PPase). Glu-20 is located in enzyme active site and apparently helps position a $\beta3$–$\beta4$ hairpin loop in WT-PPase structure by forming a hydrogen bond between the δ-carboxylate group of Glu-20 and the backbone NH of Ile-32 [8]. E20D-PPase has an appreciable hydrolytic activity (16%) [3], which indicates that Glu-20 is not essential for catalysis.

In contrast with WT-PPase, the activity of E20D-PPase measured at 25°C strongly depends on the temperature at which stock enzyme solution is preequilibrated before being

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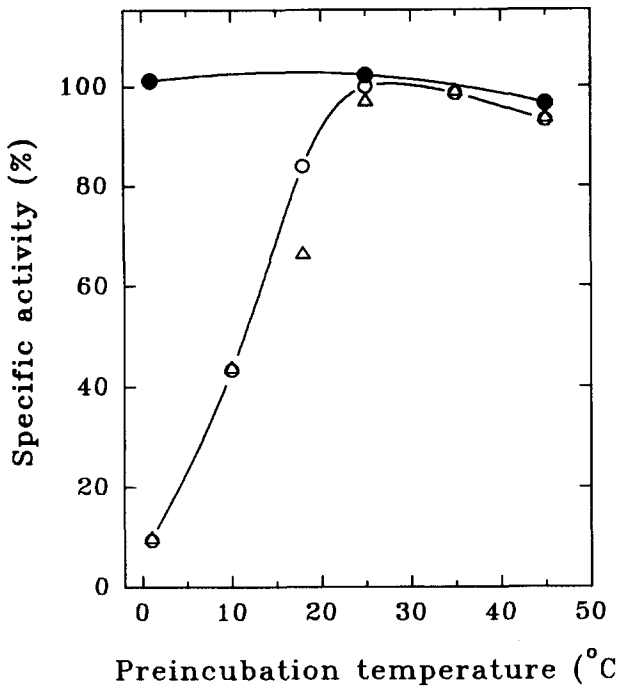

Fig. 1. Specific activities of WT-PPase (●) and E20D-PPase (○, △) preincubated at 1 mg/ml at different temperatures in the presence of 1 (●, ○) or 20 (△) mM $MgCl_2$ at pH 7.2. The activities were measured at 25°C in all cases; 100% corresponds to 63 and 450 U/mg for E20D- and WT-PPase, respectively.

added to the assay mixture (Fig. 1). The activity of the enzyme is maximal on preincubation at 25°C and above and drops to 8% at 1°C. The activities measured after 30 and 60 min of the enzyme preincubation were essentially identical, indicating that the equilibrium of the transition was attained in the stock enzyme solution at each temperature.

The effect of temperature on E20D-PPase activity is essentially independent of Mg^{2+} concentration in the stock enzyme solution (Fig. 1), ruling out the possibility that Mg^{2+} binding is impaired at low temperatures. Also, nearly identical curves were obtained at pH 6.5 and 8.5 (data not shown), indicating that the inactivation is not related to the pH change (0.028 unit/degree for the Tris-HCl buffer) caused by lowering temperature.

The kinetics of enzyme inactivation at 1°C and reactivation at 25°C are shown in Fig. 2, from which it is clear that cold inactivation can be fully reversed upon increasing temperature. The half-time of the inactivation is 8 min at both Mg^{2+} concentrations. In contrast, the reactivation rate exhibits appreciable dependence on Mg^{2+} concentration: $\tau_{1/2}$ is 0.8 and 0.4 min at 1 and 20 mM Mg^{2+}, respectively.

The activities of three PPase variants preequilibrated at 1 and 25°C are compared in Table 1. His-136 and His-140, which are substituted in the H136Q and H140Q variants, are located in the subunit contact region [6]. Incubations of H140Q-PPase were performed at a higher Mg^{2+} concentration because this variant exhibits low activity when incubated in the presence of 1 mM Mg^{2+}. The incubations were continued for at least 1 h, and constant activity levels were reached in all cases. It is seen that the equilibrium activities of all variants are greater at 25°C than at 1°C, i.e. they are cold-inactivated. However, the inactivation rates are much greater for H136Q- and H140Q-PPase ($\tau_{1/2}$<1 min) under the conditions identical to those used for Fig. 2, as compared with E20D-PPase ($\tau_{1/2}$ = 12 min). For all the variants, cold inactivation was ≥80% reversible at 25°C.

In order to test the possibility that the variant PPases are dissociated into less active units on cooling, sedimentation coefficients were measured at two temperatures (Table 1). For WT-PPase, the value of $s_{20,w}$ was close to 6 S at 1 and 25°C, indicating that this enzyme maintains its hexameric structure at both temperatures [4]. A different behavior was observed with the variant PPases. Their sedimentation coefficients measured at 25°C were close to that for hexameric WT-PPase but dropped considerably at 1°C, indicating that the molecular masses of the variants decrease in the cold. The molecular mass of H136Q-PPase at 1°C, as measured by a sedimentation equilibrium method, is 53 ± 3 kDa (pH 7.2, 0.2 mg/ml protein concentration), which is close to the predicted value of 58.7 kDa for trimeric PPase [5].

4. Discussion

The results presented indicate that the variant PPases are cold-inactivated and this effect is explained by a combination of three factors: (i) weakening of their hexameric structure with decreasing temperature; (ii) low activity of the resulting trimeric forms in the assay conditions used; (iii) low association rates in the conditions of the activity assay. That WT-PPase is not cold-inactivated is explained by the greater stability of its hexameric form. It can be dissociated only by severe treatments, such as incubations at high concentrations of urea [16] or isopropanol [17], and at low pH [18].

Hexameric structure destabilization is not unexpected on His-136 and His-140 substitutions, based on the participation of these residues in intersubunit contacts in WT-PPase [6]. However, Glu-20 is located in the active site relatively far from the subunit contact region. Thus the effect of E20D substitution on PPase quaternary structure requires a long-range structural change, consistent with the role of Glu-20 in the three-dimensional structure of WT-PPase [6,8]. That the quaternary structure of E20D-PPase is cold unstable is evidence that hydrophobic interactions are important for hexamer formation in the absence of the Glu-20–Ile-32 hydrogen bond. Analogous inferences can be made regarding the H136Q and H140Q variants.

Table 1
Temperature dependence of the activity and the sedimentation coefficient for wild-type and variant PPases at pH 7.2

PPase variant	Enzyme concentration[a] (mg/ml)	Specific activity[b] (U/mg)		$s_{20,w}$[c] (S)	
		25°C	1°C	25°C	1°C
WT	1	450	465	6.14 ± 0.10	6.02 ± 0.16
E20D	1	63	5.0	6.26 ± 0.19	3.55 ± 0.16
H136Q	0.3	475	43	5.85 ± 0.13	4.09 ± 0.14
H140Q	0.3	460	44	5.14 ± 0.10	3.71 ± 0.08

[a] In the stock enzyme solution used for activity assays and in the ultracentrifuge cell. The enzyme solutions additionally contained $MgCl_2$ at concentrations of 1 mM (WT, E20D and H136Q) or 50 mM (H140Q).
[b] The assays were initiated with the enzymes preequilibrated at the indicated temperatures and were conducted at 25°C in all cases.
[c] Values shown are the averages of two independent determinations.

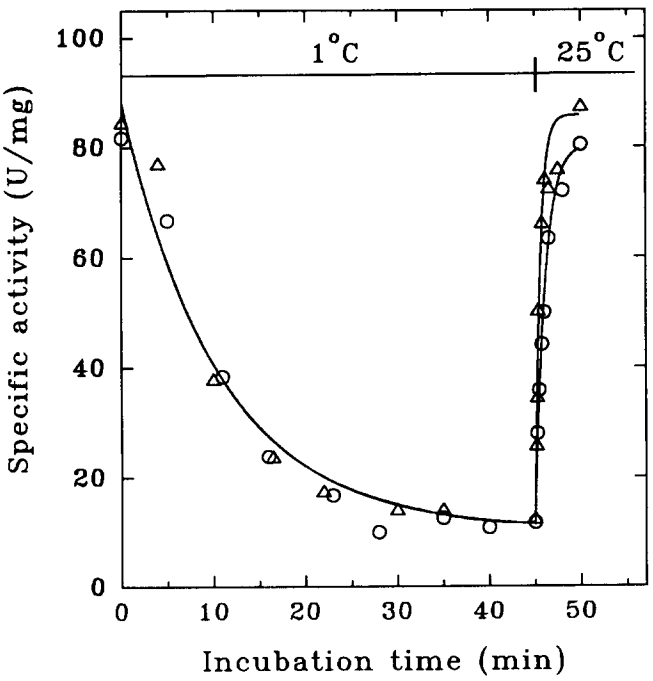

Fig. 2. Reversibility of the cold-induced inactivation of E20D-PPase in the presence of 1 (○) or 20 (△) mM $MgCl_2$. Stock enzyme solutions (1 mg/ml) preequilibrated at 25°C were rapidly chilled to 1°C and incubated for 45 min, at which time the temperature was raised back to 25°C and the incubation was continued. The pH of the incubation buffer was 7.2 at 1°C and 6.55 at 25°C.

In combination with the sedimentation data, the activity data shown in Table 1 clearly indicate that the trimeric forms of the variant PPases obtained in the cold are much less active compared to the hexameric forms. While this effect may have different explanations with different variants (low catalytic constant or weak substrate or metal activator binding), it clearly shows that intersubunit interactions somehow contribute to active site performance in spite of the fact that the corresponding regions are well separated in space. In general, it is not unreasonable to expect that the structure of a small globular protein, like *E. coli* PPase, is highly integrated rather than split into relatively independent units. These findings are, however, appear to be inconsistent with the report showing that trimeric WT-PPase prepared by prolong incubation in acidic medium is kinetically indistinguishable from hexameric WT-PPase [18].

Cold inactivation has been observed for several different classes of enzymes [19–25]. Most of them are oligomeric proteins that, like the PPase variants studied in this paper, reversibly dissociate in the cold [19,20,23–25]. Lactate dehydrogenase, by contrast, appears to unredgo a slow cold-induced conformational 'drift' without changing aggregation state [21]. A conformational change may be also responsible for the cold inactivation of a genetically engineered lysozyme [22], which is monomeric.

In conclusion, the results presented indicate that three site-directed mutants of hexameric *E. coli* PPase are subject to cold inactivation, which appears to be due to their facile dissociation into less active trimers. Such an effect of residue substitution must be given due consideration in functional studies of these and other variants of PPase.

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References

[1] Kornberg, A. (1962) in: Horizons in Biochemistry (H. Kasha and Pullman, P. eds.) pp. 251–264, Academic Press, New York.
[2] Cooperman, B.S. (1982) Methods Enzymol. 87, 526–548.
[3] Cooperman, B.S., Baykov, A.A. and Lahti, R. (1992) Trends Biochem. Sci. 17, 262–266.
[4] Wong, S.C.K., Hall, D.C. and Josse, J. (1970) J. Biol. Chem. 245, 4335–4345.
[5] Lahti, R., Pitkaranta, T., Valve, E., Ilta, I., Kuko-Kalske, E. and Heinonen J. (1988) J. Bacteriol. 170, 5901–5907.
[6] Kankare, J., Neal, G.S., Salminen, T., Glumoff, T., Cooperman, B., Lahti, R. and Goldman, A. (1994) Prot. Eng. 7, 994–1002.
[7] Oganessyan, V.Yu., Kurilova, S.A., Vorobjeva, N.N., Nazarova, T.I., Popov, A.N., Lebedev, A.A., Avaeva, S.M. and Harutyunyan, E.G. (1994) FEBS Lett. 348, 301–304.
[8] Salminen, T., Käpylä, J., Heikinheimo, P., Kankare, J., Goldman, A., Heinonen, J., Baykov, A., Cooperman, B. and Lahti, R. (1995) Biochemistry (in press).
[9] Lahti, R., Pohjanoksa, K., Pitkaranta, T., Heikinheimo, P., Salminen, T., Meyer, P. and Heinonen, J. (1990) Biochemistry 29, 5761–5766.
[10] Lahti, R., Salminen, T., Latonen, S., Heikinheimo, P., Pohjanoksa, K. and Heinonen, J. (1991) Eur. J. Biochem. 198, 293–297.
[11] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 376–382.
[12] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[13] Baykov, A.A. and Avaeva, S.M. (1981) Anal. Biochem. 1–4.
[14] Chervenka, C.H. (1972) Methods for the Analytical Ultracentrifuge, Spinco Division of Beckman Instruments Inc., Palo Alto.
[15] Chernyak, V.Ya., Magretova, N.N. and Drachev, V.A. (1982) Anal. Biochem. 123, 110–119.
[16] Wong, S.C.K., Burton, P.M. and Josse, J. (1970) J. Biol. Chem. 245, 4353–4357.
[17] Borschik, I.B., Pestova, T.V., Sklyankina V.A. and Avaeva S.M. (1986) FEBS Lett. 184, 65–67.
[18] Borschik, I.B., Sklyankina V.A. and Avaeva S.M. (1986) Biokhimiya 51, 1607–1611.
[19] Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3331.
[20] Bock, P.E. and Frieden, C. (1978) Trends Biochem. Sci. 3, 100–103.
[21] King, L. and Weber, G. (1986) Biochemistry 25, 3637–3640.
[22] Chen, B.L. and Shellman, J. (1989) Biochemistry 28, 685–699.
[23] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 3577–3582.
[24] Parry, R.V., Turner, J.C. and Rea, P.A. (1989) J. Biol. Chem. 264, 20025–20032.
[25] Huang, T.M. and Chang, G.G. (1992) Biochemistry 31, 12658–12664.