The quaternary structure of *E.coli* inorganic pyrophosphatase is not required for catalytic activity

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1. INTRODUCTION

A large amount of experimental evidence has been amassed to show that the regulation of oligomeric enzyme activity includes subunit interactions. As one looks into the regulatory role of the quaternary structure, it is essential to ascertain above all to what extent it is necessary for catalysis. One approach is to compare the oligomeric and monomeric forms of the enzyme. This problem is feasible in the case of weak subunit interactions when a mere decrease in protein concentration may suffice for dissociation. Yet most frequently, a treatment with denaturation agents like urea, guanidine hydrochloride and SDS is required. Under these conditions, even the tertiary and secondary enzymic structures are disrupted, and this does not make it possible to assay the activity of the subunits. Removal of the dissociation agents results in oligomer reconstruction, as a rule. The Chan method [1], widely used now, does not have this drawback. According to this method, an enzyme covalently coupled to a matrix via a single subunit is treated with the denaturing agents, with subsequent removal of the noncovalently bound subunits and of the denaturant. Another procedure amounts to acylation of the functional groups on the surface of the subunits upon oligomeric enzyme dissociation [2]. Yet both methods presuppose protein modification, which may impair its catalytic properties. Consequently, obtaining different molecular forms of the protein poses a difficult problem, calling for a specific solution in each particular case. Here we have elaborated an experimental procedure for mild preparation of an *E. coli* inorganic pyrophosphatase monomer. *E. coli* inorganic pyrophosphatase (EC 3.6.1.1) is a hexamer composed of identical subunits of 20 kDa each and catalyzing inorganic pyrophosphate hydrolysis in the presence of divalent metal [3].

2. MATERIALS AND METHODS

E. coli inorganic pyrophosphatase with a specific activity of 750 U/mg at pH 9.1 and 25°C was isolated from *E. coli* strain MRE-600 as in [3]. Enzyme activity was estimated as in [4].

Dissociation of the pyrophosphatase into subunits was carried out for 4 h at 25°C by incubation of the enzyme (300 μg/ml) in 0.05 M Tris-HCl, pH 7.2, containing 20% isopropanol. The activity of the pyrophosphatase subunits was determined as in [4] by addition of a small aliquot of solution (5–10 μl) to 5–10 ml assay mixture.

The sedimentation coefficients were determined as in [5]. The protein molecular mass was estimated by gel chromatography on Sephadex G-100 and by using the sedimentation equilibrium method as in [5].

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A kinetic analysis of the enzyme-induced PP_i hydrolysis in the presence of varying Mg^{2+} concentrations was studied at 25°C in 0.1 M Tris-HCl, pH 7.5, and an ionic strength of 0.1, as in [6].

3. RESULTS AND DISCUSSION

Inorganic pyrophosphatase from *E. coli* completely dissociates into monomers upon incubation in a buffer containing isopropanol. This is indicated by the following evidence: a decrease in the sedimentation coefficient, compared with the native enzyme, from 6.7–7.0 S to 2.6–3.0 S, the presence of a single protein fraction during gel chromatography with an elution volume corresponding to the protein with M_r 20000 and an M_r determined by the sedimentation equilibrium method (18000 ± 800).

Significantly, the pyrophosphatase monomer retained the full catalytic activity of the original hexamer. It was essential to determine whether this activity was due to the monomer or to a reassociated hexamer appearing as a result of the presence of the substrate or a considerable decrease (~1000-fold) in the isopropanol concentration in the enzyme activity assay. The following experiments made it possible to exclude the latter suggestion, i.e., the phenomenon of hexamer reassociation. First, the monomer activity was monitored at pH 7.2 in 20% isopropanol, amounting to 50% of the activity under optimal conditions. Second, upon incubation of the monomer using the standard assay mixture for 30 min, the sedimentation coefficient was determined; its value remained equal to 2.6 S.

To elucidate the catalytic characteristics of the pyrophosphatase monomer the hydrolysis kinetics of MgPP was studied and the main kinetic parameters of the reaction were compared with those obtained for the native enzyme. The dependence of the initial velocity of PP_i hydrolysis upon the concentration of the true substrate, MgPP, at different fixed concentrations of Mg^{2+} was studied. The MgPP concentration was varied from 7.5 to 100 μM, and that of free Mg^{2+} from 70 to 500 μM. The results of this study, plotted in double-reciprocal fashion, are shown in fig.1. To determine the number of Mg^{2+} involved in catalysis, the kinetic data were represented in Hill coordinates (not shown). The Hill coefficient was

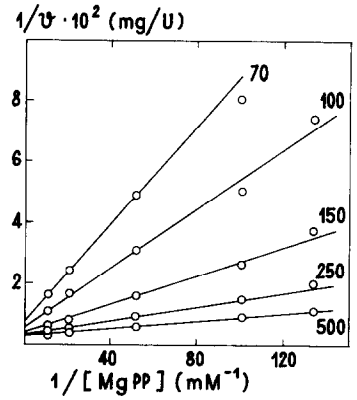

Fig.1. Dependence of the rate of PP_i hydrolysis by pyrophosphatase monomer on the concentration of MgPP at different free concentrations of Mg^{2+}. Concentrations of free Mg^{2+} are shown on the curves.

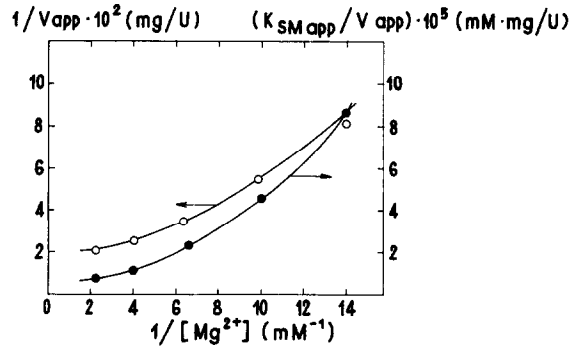

Fig.2. Dependences of $1/V_{app}$ and K_{SMapp}/V_{app} (the ordinate intercepts and the slope of the line in fig.1) on free Mg^{2+} concentration.

equal to 2.05. This indicates, as in the case of the native hexamer, that the monomer enzyme reaction involves at least two Mg^{2+}, apart from the Mg^{2+} required for the substrate to be formed. To determine the kinetic scheme of the enzymatic process, we plotted secondary dependences of the slopes (K_{SMapp}/V_{app}) and intercepts ($1/V_{app}$) from fig.1 vs $1/[Mg^{2+}]$ (fig.2). The parabolic character of these dependences accords with the previously suggested scheme for the hexamer [6].

$$\begin{array}{ccccc} E & \overset{K_A'}{\rightleftharpoons} & EM & \overset{K_A''}{\rightleftharpoons} & EM_2 \\ \| K_1 & & \| K_2 & & \| K_{SM} \\ ESM & \rightleftharpoons & EMSM & \rightleftharpoons & EM_2SM \overset{k}{\rightleftharpoons} \text{product} \end{array}$$

Table 1

E. coli inorganic pyrophosphatase hexamer and monomer characteristics

Constants	Hexamer	Monomer
M_r	120000	20000[a] 18000[b]
Sedimentation coefficient, S	6.7–7.0	2.6–3.0
V (U/mg)	570	540
K_{SM} (μM)	20.0	22.7
$K'_A K''_A$ (M^2)	8.1×10^8	8.7×10^8

[a] M_r determined by gel chromatography
[b] M_r determined by sedimentation equilibrium

This scheme corresponds to the following equation:

$$1/V = \frac{1}{V}\left(1 + \frac{K'_A K''_{SM}}{K_2[Mg]} + \frac{K'_A K''_A K_{SM}}{K_2[M]} + \frac{K'_A K''_A K_{SM}}{K_1[M]^2}\right) + \frac{K_{SM}}{[SM]V}\left(1 + \frac{K''_A}{[M]} + \frac{K'_A K''_A}{[M]^2}\right)$$

The linear character of the dependence K_{SMapp}/V_{app} and $1/V_{app}$ vs $1/[Mg^{2+}]^2$ (not shown) indicates that both in the case of the monomer and the hexamer, the content of EM and EMSM is small, i.e., the binding of the first Mg^{2+} by the monomer facilitates the binding of the second metal ion, because $K'_A \gg K''_A \cdot K'_A K''_A$ was equal to 8.7×10^{-8} M^2.

Table 1 shows the kinetic parameters of MgPP hydrolysis by the hexamer and monomer of inorganic pyrophosphatase from *E. coli*.

Thus, a study of the MgPP hydrolysis kinetics by the pyrophosphatase monomer from *E. coli* shows that the general regularities established for the native hexameric enzyme hold for the monomer as well, i.e., the complex EMg_2PPMg is catalytically active; MgPP is the true substrate, while an association of at least two Mg^{2+} with the monomer is required for hydrolysis activation; the Mg^{2+} binding affinities for the active site of both enzyme forms are probably identical. Also, K_{SM} and V_{max} practically do not change.

These results enabled us to conclude that formation of the quaternary structure is not required for the expression of the catalytic activity of inorganic pyrophosphatase from *E. coli*.

REFERENCES

[1] Chan, W.W. (1976) J. Biochem. 54, 521–527.
[2] Kasho, V.N., Bakuleva, N.P., Baykov, A.A. and Avaeva, S.M. (1982) Biokhimiya 47, 993–998.
[3] Wong, S.C.K., Hall, D.C. and Josse, J. (1970) J. Biol. Chem. 245, 4335–4340.
[4] Kurilova, S.A., Nazarova, T.I. and Avaeva, S.M. (1983) Bioorg. Khim. 9, 1032–1039.
[5] Avaeva, S.M., Plaksina, E.A. and Sklyankina, V.A. (1979) Biokhimiya 44, 1080–1083.
[6] Kurilova, S.A., Bogdanova, A.V., Nazarova, T.I. and Avaeva, S.M. (1984) Bioorg. Khim. 10, 1147–1152.