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FLUORIDE INHIBITION OF INORGANIC PYROPHOSPHATASE

III. DEPENDENCE ON THE NATURE OF SUBSTRATE AND METAL ION COFACTOR

A.A. BAYKOV, A.A. ARTJUKOV and S.M. AVAEVA

Laboratory of Bioorganic Chemistry, Moscow State University, Moscow 117234 (U.S.S.R.)

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Summary

Studies of fluoride interaction with bakers' yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) in the presence or absence of enzyme-catalyzed reactions have revealed pronounced specificity of this inhibitor. It was found that the inhibition of the enzymic hydrolysis of PP_i, ADP, ATP and tripolyphosphate in the presence of Zn^{2+} and Mn^{2+} at pH 6.5 is not time dependent and by far less extensive as that observed for the Mg^{2+}-stimulated cleavage of PP_i (apparent K_i values differ by three orders of magnitude). Addition of Ca^{2+} to the latter reaction decreases proportionally the activity of the enzyme and the rate constant for the binding of fluoride to it, which indicates that the enzyme-substrate complexes containing both Mg^{2+} and Ca^{2+} are inert in the reaction with fluoride. Preincubation of pyrophosphatase with NaF and various metal cations and substrates, in conditions where the enzyme does not act as a catalyst, does not affect its activity compared to controls lacking fluoride. The results are consistent with the proposed mechanism of mutual hindrance of substrate and fluoride release from the active site of pyrophosphatase.

Introduction

Two important observations were made in our recent work [1] on fluoride inhibition of bakers' yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) in a Mg^{2+}-PP_i assay system. First, the inhibition occurs "syncatalytically", i.e. the forward rate constant of this process depends on substrate concentration, and becomes zero at $[MgPP_i] = 0$. Second, fluoride addition to and dissociation from the enzyme proceed so slowly that their progress can be observed over several hours. In the accompanying paper [2] it has been shown that the inhibition is caused by formation of an enzyme · pyro-

phosphate · fluoride complex of 1 : 2 : 2 stoichiometry. The complex is relatively stable ($\tau_{1/2} \approx 2$ days at 4°C, pH 7.5) and not destroyed by gel filtration. Chemical studies revealed that PP_i is linked to the enzyme in this complex in a covalent manner through a β-carboxylic function of an aspartic acid residue.

This paper presents the results of kinetic studies of enzyme-fluoride interaction in the presence of various metal ions and substrates. These studies were initiated to obtain answers to the following questions:

(1) How the reaction of pyrophosphatase with NaF depends on the nature of substrate and cation activator?

(2) Is the occurance of enzymic reaction an absolute requirement for fluoride-dependent inactivation of the enzyme?

Materials and Methods

Materials

Pentasodium tripolyphosphate (grade III), disodium ADP and piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES) were obtained from Sigma Chemical Co. Imidazole and ATP (disodium salt) were purchased from Reanal. The latter substance was purified by crystallyzation from ethanol-water mixture [3]. The concentration of stock solutions of the nucleotides were determined spectrophotometrically [4]. Stock metal solutions were standardized by titration against EDTA. Other materials including pyrophosphatase were as described in the preceeding communication [1].

Kinetic measurements

A continuous enzyme assay using an automatic phosphate analyzer was employed in all kinetic studies [1]. The sensitivity of the analyzer was set at 50 μM P_i in the sample per full scale of the recorder. Rates of enzymic reactions are expressed in μmol of substrate hydrolysed per min per mg of the protein (unit/mg). It was assumed that tripolyphosphate and ATP are converted into P_i and ADP plus P_i, respectively.

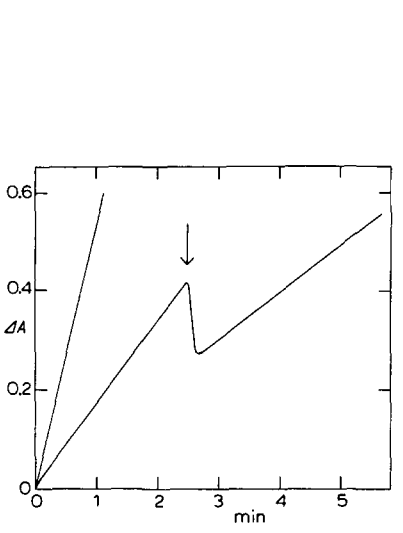
Calculation of concentrations

When enzyme activity was determined in the presence of NaF, metal concentration in the assay was increased by a factor of $(1 + K_{MF}[F])$, where K_{MF} is the stability constant of the corresponding metal · fluoride complex, and free fluoride concentration was used in subsequent calculations. The values of K_{MF} of 24, 3.4, 3.2 and 4.2 M^{-1} for MgF^+, CaF^+, ZnF^+ and MnF^+, respectively, were taken from the literature [5,6].

Results

Inhibition of pyrophosphatase reactions utilizing Zn^{2+} and Mn^{2+} as cofactors

Hydrolysis of PP_i, ATP, ADP and tripolyphosphate by bakers' yeast pyrophosphatase activated by Zn^{2+} or Mn^{2+} is inhibited by NaF. The inhibition pattern, however, changed considerably when these metals were used instead of magnesium to activate the enzyme. First, much greater concentrations of fluoride were needed to give a measurable effect on the reaction rate compared



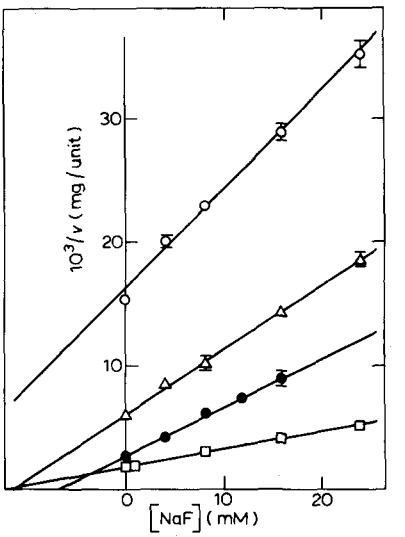


Fig. 1. Product vs. time plot for the Zn^{2+}-PP_i pair in the presence of NaF. Upper curve (control), 0.1 mM PP_i, 0.07 mM $ZnCl_2$ in 0.05 M PIPES/KOH buffer pH 6.5 at 25°C, 0.165 μg/ml enzyme. Lower curve, control in presence of 16 mM NaF; at indicated time an equal volume of the same reaction mixture but without NaF and enzyme was added.

Fig. 2. Dixon plot of initial velocity of PP_i hydrolysis with Zn^{2+} as cofactor vs. fluoride concentration at several fixed concentrations of PP_i and $ZnCl_2$. Hydrolysis rates were determined in triplicate at 25°C in 0.05 M PIPES/KOH buffer pH 6.5. The concentrations of PP_i were: 100 (filled dots) or 200 (open dots) μM, and those of $ZnCl_2$ were: ○, 40; △ and ●, 70; □, 150 μM. Cross bars indicate range of variation of measured values.

to those used in the Mg^{2+}-PP_i system experiments. Second, the fully inhibited state was instantly attained and no initial burst of product formation was observed after mixing up the reagents as is shown in Fig. 1 for the Zn^{2+}-PP_i pair. The extent of inhibition was the same regardless of the order of addition of the inhibitor and substrate.

TABLE I

APPARENT INHIBITION CONSTANT FOR FLUORIDE IN VARIOUS SUBSTRATE-COFACTOR SYSTEMS

The values of K_F were obtained from plots analogous to that shown in Fig. 2 by the least squares method. NaF concentration was 0—30 mM. Controls run with 30 mM NaCl showed no inhibition by the sodium ion. Other reaction conditions were the same as in Fig. 2.

No.	Activating cation	Substrate	K_F (mM)
1	1 mM Mg^{2+}	0.5 mM PP_i	0.013 ± 0.01
2	0.04 mM Zn^{2+}	0.2 mM PP_i	20 ± 1.5
3	0.07 mM Zn^{2+}	0.2 mM PP_i	11.7 ± 0.6
4	0.15 mM Zn^{2+}	0.2 mM PP_i	12.2 ± 0.4
5	0.07 mM Zn^{2+}	0.1 mM PP_i	6.6 ± 0.7
6	0.07 mM Mn^{2+}	0.1 mM PP_i	5.5 ± 0.4
7	0.5 mM Zn^{2+}	0.2 mM tripoly-phosphate	33.5 ± 6
8	0.5 mM Zn^{2+}	0.2 mM ATP	100 ± 20
9	1.5 mM Zn^{2+}	1.0 mM ATP	33 ± 7
10	0.5 mM Zn^{2+}	0.2 mM ADP	85 ± 25

Dilution experiments (Fig. 1) also attested to the reversibility of the interaction of fluoride and inorganic pyrophosphatase. The enzyme activity was increased from 32 to 43.5% when the concentration of the inhibitor was quickly decreased from 16 to 8 mM by dilution into an equal volume of a solution containing buffer, substrate and metal activator *. When pyrophosphatase was directly assayed at 8 mM NaF, without preincubation at 16 mM fluoride, its activity was 42%. These observations are just what would be expected if fluoride is a reversible inhibitor.

The dependence of the enzyme-catalyzed reaction rate on fluoride concentration at fixed substrate and cofactor concentrations obeyed Eqn. 1 for all substrate-cofactor pairs listed in Table I.

$$\frac{1}{v} = \frac{1}{v_0}\left(1 + \frac{[F]}{K_F}\right) \quad (1)$$

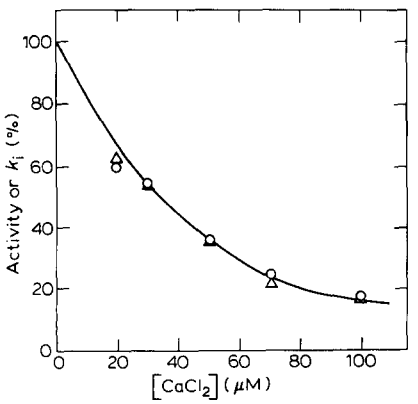
The apparent inhibition constant K_F can be determined from a $1/v$ versus [F] graph (Dixon plot). It is known that the physical meaning of this parameter depends on the type of inhibition [7]. The results (Fig. 2) do not comply with any of the simple kinetic model of inhibition since the lines are neither parallel nor they have a common intersection point. The usefulness of the Dixon plot may be limited in this case by the fact that the mixture of PP_i and a divalent cation contains at least two hydrolyzable substrate species (MPP_i and M_2PP_i) as has been shown by Moe and Butler [8] for the Mg^{2+}-dependent enzymic cleavage of PP_i. Clearly, the amounts of the two probable enzyme-substrate complexes do not change in parallel when the concentrations of added PP_i and metal are varied in some casual way, and therefore the enzymic reaction cannot be treated as a single-substrate one. It is seen that under the conditions where the concentration of the 2 : 1 complex is expected to be low (high $[PP_i]/[Zn^{2+}]$ ratio), the inhibition approaches uncompetitive type (the nomenclature of Cleland [9] is used) which was observed earlier [1] in the Mg^{2+}-PP_i system.

The apparent inhibition constant K_F for the Mg^{2+}-stimulated hydrolysis of PP_i is about three orders of magnitude lower than that for other studied substrate-cofactor pairs (Table I). Although the exact meaning of K_F remains uncertain, one tends to ascribe the observed difference in this parameter to the difference in the strength of the inhibitor binding to the enzyme.

Fluoride inhibition of pyrophosphatase in the Mg^{2+}-Ca^{2+}-PP_i system

It is known from previous studies [10] that the complex of this enzyme with pyrophosphate contains three catalytically important sites of interaction with Mg^{2+} and Ca^{2+} and the binding of Ca^{2+} even to one of them blocks the enzymic reaction. This phenomenon was used to study the interaction of fluoride with the mixed complexes formed by substrate-enzyme intermediate with the two alkali earth metals.

* For comparison with the rates before dilution, the rates after dilution must be doubled in order to account for the twofold decrease in enzyme concentration.



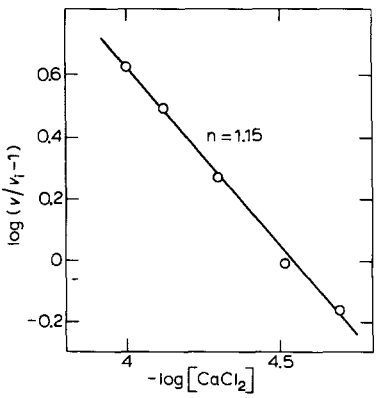


Fig. 3. Initial velocity of PP_i hydrolysis (○) and rate constant of fluoride binding to pyrophosphatase (△) vs. Ca^{2+} concentration. The assays contained 0.1 M imidazole · HCl buffer pH 6.5 at 25°C, 1.0 mM $MgCl_2$, 0.5 mM PP_i and indicated concentrations of $CaCl_2$. The inhibition rate constant k_i was estimated as described in the preceeding paper [1]. The values of k_i and enzyme activity at $[CaCl_2] = 0$ (26.6 $M^{-1} \cdot s^{-1}$ and 406 unit/mg, respectively) were taken for 100%. Variations in measured values were within the size of the symbols.

Fig. 4. Hill plot of initial velocity of PP_i hydrolysis vs. Ca^{2+} concentration. The data were taken from Fig. 3.

It was found that Ca^{2+} slows down the rate of the attainment of the fully inhibited state of the enzyme. Fig. 3 shows the effect of Ca^{2+} on the activity of pyrophosphatase and the rate constant of fluoride addition to the enzyme. The latter parameter was determined using a linearized $[P_i]$ versus time plot for the enzyme-catalyzed hydrolysis of PP_i in the presence of NaF as described earlier [1]. It is seen that two curves in Fig. 3 coincide. Therefore, the catalytically inactive enzyme · substrate · Mg^{2+} · Ca^{2+} complexes formed in these conditions do not bind fluoride. The backword rate constant for enzyme-fluoride interaction k_r [1] was independent of $[Ca^{2+}]$ and equal to $3.5 \pm 0.3 \cdot 10^{-4}$ s^{-1}.

The data on the inhibition of enzymic reaction by Ca^{2+} were further analyzed using a Hill plot (Fig. 4). The value of the Hill coefficient was found to be near unity. This means that the inhibitory cation replaces only one Mg^{2+} ion in the enzyme · substrate complex over the range of Ca^{2+} concentrations employed in this experiment. This is in agreement with our earlier observation [10] that the affinity of one of the sites for Ca^{2+} considerably exceeds that of the others.

Fluoride-enzyme interaction in the absence of hydrolytic reaction

It was found earlier [2] that full restoration of the pyrophosphatase activity inactivated by NaF in the presence of Mg^{2+} and PP_i takes several hours at 25°C after removal of the excess of the inhibitor. We kept in mind this property of the enzyme in searching for occurance of inactivation by NaF in several systems where no hydrolytic reaction occurs. In these experiments, the enzyme was incubated with fluoride and various additives at pH 6.5 for 30 min and its activity was measured thereafter in the Mg^{2+}-PP_i system. Fluoride added into the assay mixture with pyrophosphatase did not affect initial rates because of great dilution. The results are summarized in Table II. It is seen that only

TABLE II

EFFECT OF SOME REAGENTS ON THE ACTIVITY OF PYROPHOSPHATASE BY ITS INCUBATION WITH NaF

The enzyme (4.1 μg) was incubated in a total volume of 0.25 ml at 25°C in 0.05 M PIPES/KOH buffer pH 6.5 containing the indicated reagents. NaF concentration was 10 mM, except for experiments with Ca^{2+} where CaF_2 precipitation was observed at this level of fluoride. In 30 min a 90-μl aliquot was withdrawn and assayed for enzyme activity of 25°C in 0.1 M imidazole · HCl buffer pH 6.5 containing 3 mM $MgCl_2$ and 1 mM PP_i. The results are given as the means of 3—5 separate experiments ± S.E. The activity of pyrophosphatase before incubation was taken for 100%

No.	Additives	% residual activity	
		—NaF	+NaF
1	2 mM $MgCl_2$ + 0.5 mM PP_i	104 ± 2.0	1.2 ± 0.5
2	—	88 ± 2.0	92 ± 3.0
3	5 mM $MgCl_2$	112 ± 1.0	105 ± 5.5
4	5 mM $ZnCl_2$	74.5 ± 3.5	72 ± 3.0
5	1 mM $CaCl_2$	72 ± 2.0	75.5 ± 1.0 *
6	5 mM PP_i	88.5 ± 1.5	84.5 ± 1.0
7	5 mM P_i	93 ± 2.5	86 ± 4.5
8	1 mM $CaCl_2$ + 0.5 mM PP_i	82.5 ± 1.0	81.5 ± 0.5 *
9	5 mM $MgCl_2$ + 5 mM P_i	119 ± 1.5	83.5 ± 1.5
10	5 mM $MgCl_2$ + 5 mM ATP	75.5 ± 4.5	71.5 ± 2.0

* [NaF] = 1 mM.

PP_i and P_i in the presence of $MgCl_2$ promote fluoride-dependent inactivation of the enzyme. Since in both systems enzymic hydrolysis or synthesis of PP_i occurs, this finding may indicate that the pyrophosphorylated enzyme is formed in both reactions.

As is also seen from Table II, the incubation of pyrophosphatase for 30 min at 25°C without NaF resulted in a decrease in enzyme activity by 10—25% in some cases. This inactivation is at least partly accounted for by the fact that the activity of the enzyme was estimated from initial velocity of PP_i hydrolysis, while the progress curves, obtained after preincubation in Expts. 2, 4—6 and 8, were clearly concave upward. Such behaviour can be explained by slow conformational changes of the protein molecule induced by cations [11]. This conclusion is supported by the observation of strict linearity of kinetic curves after incubation in mixtures containing Mg^{2+}.

Discussion

It was shown in the accompanying paper that the inhibition of pyrophosphatase by fluoride is explained by formation of a stable enzyme · PP_i · F complex, in which PP_i is covalently bound to the enzyme. To account for the unusual stability of this association we proposed a mechanism of simultaneous binding of fluoride and PP_i to the active site of the enzyme. It was assumed that the binding occurs in such a manner that fluoride blocks the step of the enzymic reaction following pyrophosphorylation while the covalently bound molecule of PP_i causes steric hindrance of fluoride release. The reactivation of pyrophosphatase can then be achieved bo coming back from the covalent intermediate with PP_i to a michaelian one.

This mechanism predicts that the quantitative characteristics of the inhibition be sensitive to the nature of the metal · substrate complex, which is the actual substrate for this enzyme. The results reported above comply with this prediction. It was found that fluoride inhibition of Zn^{2+}- and Mn^{2+}-stimulated pyrophosphatase reactions is neither time dependent nor as extensive as the inhibition observed in the Mg^{2+}-PP_i system. This difference indicates that the apparent rate constant for the decomposition of enzyme · fluoride complex, k_r, is considerably enhanced when Zn^{2+} or Mn^{2+} is used as cofactor instead of Mg^{2+}. Really, the absence of a product burst in the kinetic curve obtained by our continuous method on addition of the enzyme to the reaction mixture containing about 10 mM NaF shows that the apparent rate constant for fluoride addition to the enzyme, k_i, is higher than 10 $M^{-1} \cdot s^{-1}$. Therefore, k_r, which is equal to $k_i K_F$, is larger than 0.1 s^{-1} for the Zn^{2+}- and Mn^{2+}-stimulated reactions. Comparing, the corresponding values for the Mg^{2+}-PP_i pair in the same conditions are: $k_i = 26.7\ M^{-1} \cdot s^{-1}$, $k_r = 3.5 \times 10^{-4}\ s^{-1}$ (Fig. 3 and text).

A similar effect of the nature of substrate on the mode of inhibition by fluoride was reported by Haugen and Suttie [12] for carboxylesterase.

The absence of the inactivation of pyrophosphatase under the conditions where no pyrophosphorylation of the enzyme occurs, also contributes to give further credit to the proposed mechanism of fluoride action. The observed inactivation in the Mg^{2+}-P_i system is not unexpected because both the breakdown and synthesis of PP_i must proceed through the same pyrophosphoryl enzyme intermediate.

The inertness of the enzyme · substrate complex in which one Mg^{2+} ion is replaced by Ca^{2+} in the reaction with NaF can shed some light on the mechanism of the inhibition of enzymic reaction by Ca^{2+}. The binding of Ca^{2+} to the enzyme inactivated by fluoride in the Mg^{2+}-PP_i system appears not to accelerate its reactivation since k_r does not depend on $[Ca^{2+}]$. One can therefore, conclude that Ca^{2+} blocks the pyrophosphorylation step or some step before it in the hydrolytic reaction sequence.

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