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FLUORIDE INHIBITION OF INORGANIC PYROPHOSPHATASE

I. KINETIC STUDIES IN A Mg^{2+}-PP_i SYSTEM USING A NEW CONTINUOUS ENZYME ASSAY

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Summary

Reversible inhibition of bakers' yeast inorganic pyrophosphatase (EC 3.6.1.1) by fluoride has been studied as a function of substrate, metal-ion activator and inhibitor concentrations and pH using a new continuous enzyme assay with an automatic phosphate analyzer. The inhibition was shown to be the result of tight binding of fluoride by two catalytically active enzyme-substrate complexes. The reaction between pyrophosphatase and fluoride is relatively slow, so that the rate constants for the binding and release of the inhibitor were derived from phosphate formation curves measured on the time scale of enzyme assays. The pH-dependence of the inhibition reaction in the alkaline medium indicates that both the fluoride-enzyme interaction and the catalytic step of the pyrophosphatase reaction are controlled by the same group on the protein. In the acidic medium, the inhibition is considerably enhanced, presumably because of the protonation of another enzyme group.

Introduction

Examples of strong inhibition of enzymic reactions by fluoride are both copious and of long standing in enzymology [1,2]. Most of the available data, however, concern the enzymes containing within their structure transition-row metals, and relatively little is known about the nature of fluoride effect on the enzymes of other types. In order to gain insight into the mechanism of fluoride interaction with protein catalysts activated by alkali earth metals, we have been carrying out a detailed study of the inhibition of the reaction catalyzed by bakers' yeast inorganic pyrophosphatase (EC 3.6.1.1). This magnesium-acti-

vated enzyme can be obtained in a highly purified state and appears to satisfy the requirement for being well characterized. Recent studies of yeast pyrophosphatase in several laboratories have revealed the main features of its structure and mechanism, including partial amino acid sequence [3,4], quaternary structure [5—7], the nature of activation by metals [8—11] and the participation of a phosphoryl-enzyme intermediate [12]. The enzyme is strongly inhibited by fluoride [13].

This paper describes some kinetic studies of fluoride inhibition of inorganic pyrophosphatase in a Mg^{2+}-PP_i assay system.

Materials and Methods

Materials

Inorganic pyrophosphatase was isolated from bakers' yeast by the method of Cooperman et al. [14]. Specific activity of the preparation at 30° C, pH 7.2 was 1050 units/mg (65 Kunitz units/mg, Ref. 15). The concentrations of the enzyme solutions were determined assuming an absorbance coefficient $A_{280}^{0.1\%} = 1.45$ [15] and a molecular weight of 70 000 [5,6].

All buffers were obtained from Sigma Chemical Co. Disodium fluorophosphate was prepared according to a method adopted from the literature [16]. Other chemicals were obtained from Reachim and were of the highest available purity.

Kinetic measurements

A continuous assay was developed to follow substrate hydrolysis with the phosphate analyzer described earlier [9,17]. The relay programmer of the analyzer was disconnected, and the reaction mixture was continually pumped off at a flow rate of 2.5 ml/min. Dye and detergent concentrations of the dye solution were increased to 0.13 and 1.2 g/l, respectively, and a delay coil made of 0.12 ×400 cm Teflon tubing was incorporated in the manifold between the mixing device and the photometer to prevent formation of a blue film on the walls of the photometer cuvette. With this modification, the analyzer could be used to monitor phosphate content for at least 10 min before washing out was needed. An absorbance change of 0.5 unit (corresponding to 125 μM P_i) was displayed full scale on a LKB 6500 flat-bed recorder, and a change of 1 μM P_i in the reaction medium could be easily resolved.

The enzymic reaction was initiated by adding PP_i to otherwise complete mixtures in a 30-ml water-jacketed and magnetically stirred cell. Continuous recording of kinetic curves was usually carried out for the first 5 min of the reaction only. Further progress was followed by starting the analyzer for 1 min at 5-min intervals. Rates of pyrophosphate hydrolysis in controls lacking enzyme were negligible.

All the experiments were performed at 25° C.

Paper electrophoresis

This was done as described by Di Sabato and Jencks [18].

Treatment of kinetic data

The data were fitted to the equation of a straight line by least-squares computation.

Results

Effect of anions on enzyme activity

Seven sodium salts, including NaF, were tested for their ability to change the rate of the enzyme-catalyzed hydrolysis of pyrophosphate. The results (Fig. 1) show that all the salts are inhibitory and that the extent of inhibition depends on the nature of their anionic part. A two-fold increase in Mg^{2+} concentration in the assay diminished the inhibition caused by all salts, with the exception of NaF.

The effect of the salts can be explained simply by the decrease in the activities of reactants as the ionic strength increases and by complexation of the metal-ion activator and substrate with the anions and cation of the added electrolytes. These trivial mechanisms, however, cannot be applied to fluoride since its effect was observed at low salt concentration and showed little dependence on the activator level.

Fluorophosphate, a probable product of the pyrophosphatase reaction in the presence of fluoride, was not inhibitory at a concentration of 1 mM in these conditions.

Reaction products in the presence of fluoride

Inorganic pyrophosphatase had been added to the mixture containing PP_i,

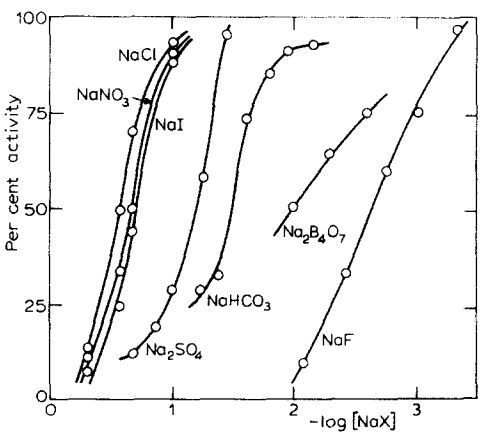


Fig. 1. Effect of sodium salts on the activity of inorganic pyrophosphatase. The assays contained 50 μM pyrophosphate, 100 μM $MgCl_2$, $1.4 \cdot 10^{-10}$ M enzyme and 0.05 M Tris · HCl (pH 8.0) in a total volume of 3 ml. The reaction was run for 5 min and was stopped by the addition of 50 μl H_2SO_4 (2.5 M). Phosphate released was measured with an analyzer.

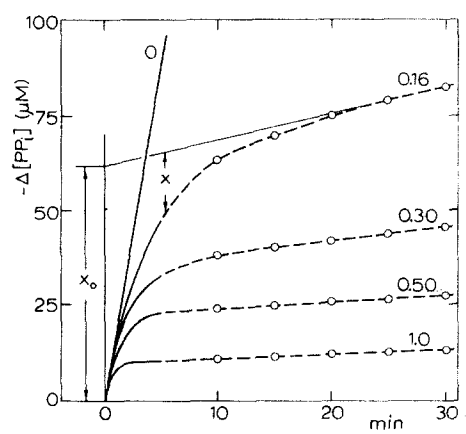


Fig. 2. Product formation curves of pyrophosphatase reaction in the absence and in the presence of fluoride. The assays contained 0.5 mM pyrophosphate, 2 mM $MgCl_2$, $3.15 \cdot 10^{-10}$ M enzyme and 0.1 M Tris · HCl (pH 7.5). The parts of the curves shown by solid lines were recored in a continuous way. Further details can be found in Methods. Fluoride concentration (mM) is indicated at each curve. Values of x and x_0 (see text) are shown only for the curve obtained at 0.16 mM fluoride.

$MgCl_2$ and NaF, all at a concentration of 10 mM, and 0.05 M Tris · HCl (pH 8.0) until approximately a half of the substrate was converted into P_i. Paper electrophoresis showed orthophosphate was the only detectable product of the enzymic reaction.

Time-course of the inhibition by fluoride

Product-formation curves of the pyrophosphatase reaction show a transient phase in the presence of fluoride (Fig. 2). Addition of new portions of the substrate and/or activator after onset of final velocity did not significantly change the rate of phosphate formation whereas addition of the enzyme brought about a new burst of product formation (not shown in Fig. 2) similar in shape to the initial one. Dialysis of the fluoride-inhibited enzyme against water resulted in complete restoration of activity.

The apparent enzyme inactivation could be described by Eqn. 1.

$$[E]_{active} + F \underset{k_r}{\overset{k_i}{\rightleftharpoons}} [E]_{inactive} \tag{1}$$

If (a) the hydrolytic reaction proceeds linearily in the absence of fluoride, (b) the inactivation reaction is slow and does not affect the equilibria of the enzyme-substrate-activator reaction, and (c) the inhibitor is in great excess over the enzyme, the following system of three equations is valid

$$-\frac{d[PP_i]}{dt} = k[E]_{active} \tag{2}$$

$$\frac{d[E]_{active}}{dt} = k_r[E]_{inactive} - k_i[F][E]_{active} \tag{3}$$

$$[E]_{active} + [E]_{inactive} = [E]_0 \tag{4}$$

where $[E]_0$ is the total enzyme concentration and k is the constant relating the rate of the catalytic reaction to $[E]_{active}$. The system can be solved to give Eqn. 5, describing the time-course of pyrophosphate hydrolysis in the presence of fluoride.

$$-\Delta[PP_i] = k[E]_0\left(\frac{k_r}{k_i[F] + k_r}\, t + \frac{k_i[F]}{(k_i[F] + k_r)^2}\,[1 - e^{-(k_i[F] + k_r)t}]\right) \tag{5}$$

After appropriate rearrangements, Eqn. 2 can be used to determine rate constants k_i and k_r graphically from kinetic recordings shown in Fig. 2. At large t, Eqn. 5 can be reduced to:

$$-\Delta[PP_i] = k[E]_0\left(\frac{k_r}{k_i[F] + k_r}\, t + \frac{k_i[F]}{(k_i[F] + k_r)^2}\right) \tag{6}$$

Extrapolation of this linear function to zero time gives a positive ordinate intercept:

$$x_0 = k[E]_0\,\frac{k_i[F]}{(k_i[F] + k_r)^2} \tag{7}$$

Introducing a new variable (see Fig. 2)

$$x = k[E]_0 \frac{k_i[F]}{(k_i[F] + k_r)^2} e^{-(k_i[F] + k_r)t} \quad (8)$$

one can obtain from Eqns. 7 and 8 that

$$\log \frac{x_0}{x} = 0.434 (k_i[F] + k_r)t \quad (9)$$

As can be seen from Eqn. 9, plots of log (x_0/x) vs. time, at various inhibitor concentrations, will form a family of straight lines with slopes equal to $(k_i[F] + k_r)$. Such a plot is shown in Fig. 3a for the data obtained at pH 7.5. The k_i constant is easily obtained by replotting the slopes vs. fluoride concentration (Fig. 3b). The value of k_r was too small and could not be determined from the same graph. This constant was derived from a plot of k/k_∞ vs. [F] (Fig. 4) according to Eqn. 10,

$$\frac{k}{k_\infty} = 1 + \frac{k_i}{k_r}[F] \quad (10)$$

where k_∞ is the rate constant of the pyrophosphatase reaction after onset of the quilibria of the fluoride-enzyme reaction. The computed values of the kinetic parameters at pH 7.5 were $k_i = 24 \pm 1$ $M^{-1}s^{-1}$ and $k_r = (1.60 \pm 0.1) \cdot 10^{-4}s^{-1}$.

In the experiments described below, kinetic parameters for fluoride-dependent enzyme inactivation under each set of conditions were determined from separate continuous recordings of product formation according to Egns. 9 and 10. The recordings were obtained in duplicate at two fluoride concentrations such that $(k_i[F] + k_r)$ ranged from 0.3 to 1 min^{-1}. The k constant was determined according to Eqn. 2 at [F] = 0. The results are given as the means of four determinations ± S.E. The rate constant k_i was calculated in terms of free fluoride concentration using the dissociation constant $K_d = 48$ mM for the MgF^+ complex [19].

Part of the inhibitory effect exerted by fluoride at low Mg^{2+} content could

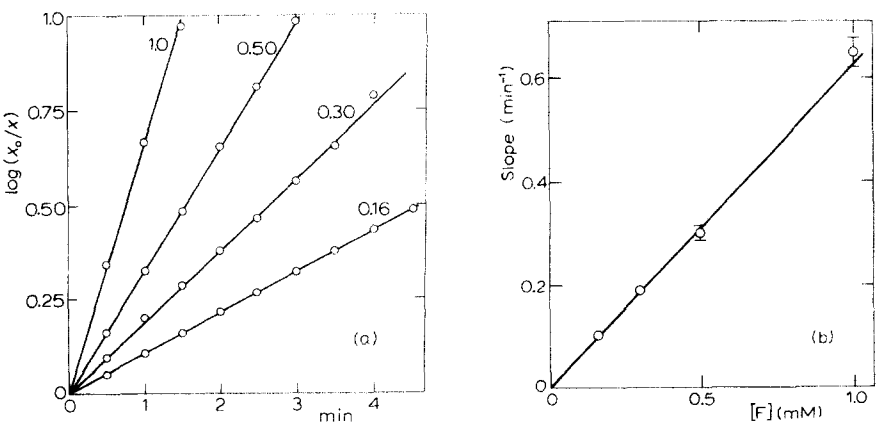
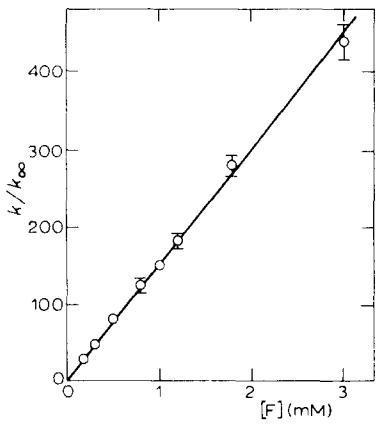


Fig. 3. (a) Data of Fig. 2 replotted according to Eqn. 6. The numbers by the lines refer to fluoride concentration in mM. (b) Dependence of the slope of the lines in (a) on fluoride concentration.



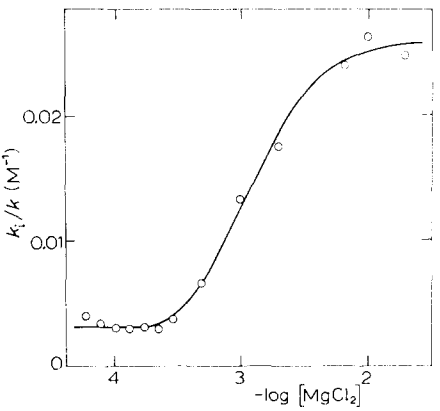


Fig. 4. Dependence of the k/k_∞ value on fluoride concentration. Conditions were as described in Fig. 2.

Fig. 5. Dependence of the k_i/k value on activator concentration as calculated from the data in Table I.

be attributed to the sequestration of the activating cation. This contribution in the inhibition was eliminated by increasing the concentration of $MgCl_2$, compared to that used for the measurement of k, by a factor of $(1 + [F]/K_d)$.

Enzyme species reacting with fluoride

The presence of metal activator and substrate appears to be an absolute requirement for inactivation of pyrophosphatase by fluoride. A 30-min pretreatment of the enzyme with 10 mM fluoride in the same conditions as for Fig. 2, but without $MgCl_2$ or without PP_i or with neither did not result in any change in product-formation curve compared to control (identical pretreatment, but without fluoride). This means that under the conditions of the preincubation, k_i is considerably smaller and/or k_r is considerably larger than in the complete mixture.

Recent kinetic studies of the pyrophosphatase-catalyzed reaction [8—11] have revealed the following regularities of enzyme-substrate interactions. The enzyme must be activated by free Mg^{2+} before binding the substrate. Two different metal-substrate complexes, $MgPP_i$ and Mg_2PP_i, are hydrolyzed at different rates, and uncomplexed PP_i is a strong competitive inhibitor. The rate constant of the pyrophosphatase-catalyzed reaction k is given by Eqn. 11.

$$k = \frac{k'[ES_1] + k''[ES_2]}{[E]_0} \tag{11}$$

where $[ES_1]$ and $[ES_2]$ refer to the complexes of the preactivated enzyme with $MgPP_i$ and Mg_2PP_i and k' and k'' refer to the catalytic constants for these complexes, respectively.

The inactivation reaction appears also to involve only the catalytically active enzyme-substrate intermediates, and the rate constant k_i can be related to their concentrations by Eqn. 12,

$$k_i = \frac{k_1'[ES_1] + k_i''[ES_2]}{[E]_0} \tag{12}$$

where k_i' and k_i'' are second-order rate constants for the reaction between fluoride and respective enzyme species. Dividing Eqn. 12 by Eqn. 11 yields Eqn. 13.

$$\frac{k_i}{k} = \frac{k_i'[ES_1] + k_i''[ES_2]}{k'[ES_1] + k''[ES_2]} \tag{13}$$

which predicts that the k_i/k ratio will become constant at very low and very high levels of the activator where either $[ES_1]$ or $[ES_2]$ can be neglected. Table I and Fig. 5 show that experimental data satisfy this requirement.

The analysis of the distribution of the enzyme among various species using the equilibrium constants measured at pH 8.6 [11] shows that a considerable part of it exists at the low level of the activator as a catalytically inactive complex with free pyrophosphate. The proportionality between k_i and k in these conditions indicates that this species is not attacked by fluoride.

The inactivation rate constant k_i'' appears to be larger than k_i'. In fact, one can see from Fig. 5 that k_i''/k'' is 8—9 times as large as k_i'/k', whereas k'/k'' (estimated, however, in somewhat different conditions, [10], is equal to 3—4.

Dependence of inhibition on pH

The kinetic parameters describing the inhibition of pyrophosphatase by fluoride were measured at a fixed substrate concentration as a function of pH (Table II). The activator concentration was chosen so that the $[Mg_2PP_i]/[MgPP_i]$ ratio was less than 0.02 [20] and the terms containing $[ES_2]$ in Eqn. 13 could be neglected. It was shown above at pH 8.5 that Eqn. 13 becomes in this case

$$\frac{k_i}{k} = \frac{k_i'}{k'} \tag{14}$$

The independence of k_i/k on activator concentration within an 0.5—1.0 mM

TABLE I

DEPENDENCE OF ENZYME ACTIVITY AND OF RATE CONSTANTS FOR FLUORIDE INHIBITION OF PYROPHOSPHATASE ON ACTIVATOR CONCENTRATION

Kinetic parameters ± S.E. were determined at 25°C as described in the text in assay mixtures containing 0.5 mM PP_i, 0.4—10 mM NaF, 0.1 M Tris · HCl (pH 8.5) and various concentrations of $MgCl_2$.

$[MgCl_2]$ (mM)	k (s^{-1})	k_i ($M^{-1}\ s^{-1}$)	$k_r \cdot 10^4$ (s^{-1})
0.061	175 ± 8	0.70 ± 0.05	1.15 ± 0.06
0.080	222 ± 5	0.73 ± 0.04	1.08 ± 0.07
0.104	368 ± 13	1.11 ± 0.03	1.25 ± 0.04
0.135	500 ± 10	1.47 ± 0.07	1.24 ± 0.09
0.175	697 ± 7	2.15 ± 0.10	1.05 ± 0.05
0.23	840 ± 16	2.45 ± 0.11	1.15 ± 0.08
0.295	880 ± 10	3.25 ± 0.20	1.10 ± 0.15
0.50	906 ± 8	6.0 ± 0.25	1.25 ± 0.10
1.0	754 ± 13	10.0 ± 0.5	2.05 ± 0.15
2.0	664 ± 21	11.7 ± 0.6	3.05 ± 0.20
5.0	594 ± 11	13.7 ± 0.4	3.90 ± 0.30
10.0	572 ± 8	15.0 ± 0.9	3.90 ± 0.40
20.0	524 ± 8	13.0 ± 0.4	4.00 ± 0.20

TABLE II

DEPENDENCE OF ENZYME ACTIVITY AND OF RATE CONSTANTS FOR FLUORIDE INHIBITION OF PYROPHOSPHATASE ON pH

Kinetic parameters ± S.E. were determined at 25°C as described in the text in assay mixtures containing 1 mM PP_i, 0.5 mM (at pH > 6.0) or 1.0 mM (at pH ≤ 6.0) $MgCl_2$, 0.4—4 mM NaF, 0.1 M buffer and KCl to ionic strength of 1.0.

pH	Buffer *	k (s^{-1})	k_i ($M^{-1}\ s^{-1}$)	$k_r \cdot 10^4$ (s^{-1})
5.4	A	0.86 ± 0.02	2.6 ± 0.15	6.75 ± 0.03
5.7	A	3.45 ± 0.09	5.25 ± 0.40	6.8 ± 0.4
6.0	A	33.0 ± 0.6	10.0 ± 0.25	7.5 ± 0.5
6.2	B	33.5 ± 0.7	6.6 ± 0.15	6.8 ± 0.2
6.7	B	117 ± 2	10.7 ± 0.4	5.15 ± 0.3
7.1	B	130 ± 3	7.9 ± 0.3	4.55 ± 0.3
7.6	B	217 ± 7	8.5 ± 0.25	2.8 ± 0.2
7.4	C	220 ± 5	6.7 ± 0.7	3.5 ± 0.25
8.0	C	194 ± 5	5.3 ± 0.25	3.3 ± 0.3
8.4	C	173 ± 6	5.1 ± 0.15	4.25 ± 0.5
9.0	C	139 ± 4	3.95 ± 0.25	4.7 ± 0.35
9.4	C	85 ± 1	2.85 ± 0.5	5.3 ± 0.3

* Buffers used: A, 2-(*N*-morpholinoethanesulfonic acid)/KOH; B, imidazole/HCl; C, Tris · HCl.

range at 0.5 mM PP_i at pH 6.0 and an ionic strength of unity, suggests that Eqn. 14 holds also in the acidic pH region.

The pH profile of k' can conveniently be obtained from that of k_i/k using the available data on the effect of pH on k'.

The lack of effect of pH on k_i/k at pH > 7.5 (Fig. 6) indicates that k_i' exhibits a pH dependence analogous to that of k'. Previous results [10,11,21] suggest that the catalytic step of the pyrophosphatase reaction requires a protonated enzyme group with pK_a near 8.7. It is likely that the inactivation reaction also depends on the ionization state of this group.

The increase in k_i/k at higher proton concentration is certainly due to k_i' because k' is essentially constant in a pH range of 5.4—7.5 (Ref. 21, and unpublished results of A.A. Baykov and S.M. Avaeva). The pK_a of the group affecting the enzyme-fluoride interaction in the acidic medium could not be determined because of enzyme instability at a pH below 5.4.

The reactivation rate constant k_r also changes with pH and is minimal at a pH near to 7.6 (Table II).

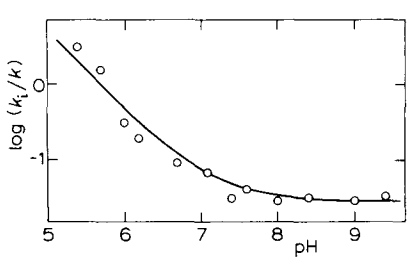


Fig. 6. Dependence of the k_i/k value on pH as calculated from the data in Table II.

The enzyme appeared to be quite stable in the conditions of these experiments, as judged by the strict linearity of the enzymic reaction both in the absence of fluoride and after onset of fluoride-enzyme equilibrium. A similar pH stability pattern was obtained by Cooperman and Chiu [22]. It should be noted that the pyrophosphatase isolated from yeast by a different purification procedure is considerably less stable in the acidic pH region [23].

Control experiment

Precipitation of MgF_2 (solubility product, $10^{-8.2}$ [24]) was expected for some of the used assay mixtures but never observed. It seems that this species is formed very slowly. The following experiment substantiates this conclusion. Solid NaF was added to a final concentration of 0.01 M to a solution of $MgCl_2$ (0.01 M) in 0.05 M piperazine-*N*,*N*′-bis(ethanesulfonic acid)/NaOH buffer (pH 6.5), and the Mg^{2+} content was monitored using a metal-sensitive electrode (model 92–32, Orion Research, Inc.). The concentration of the Mg^{2+} ion was found to remain constant for at least 1 h after a rapid decrease by 20%, which can be accounted for by formation of MgF^+ [19].

Discussion

The results reported above show that the introduction of fluoride in the assay mixture results in the reversible inhibition of inorganic pyrophosphatase. Two unique futures of the inhibition should be kept in mind by the deduction of a plausible mechanism of it. First, the enzyme-fluoride reaction proceeds "syncatalytically", i.e. it requires the simultaneous presence of the substrate and the metal-ion cofactor. Furthermore, the rate laws describing the interaction of the enzyme with fluoride and the enzyme-catalyzed hydrolysis of the substrate are similar to each other, and changes in the metal content of the bound substrate species or in the ionization state of the essential enzyme group with a pK_a of 8.7 affect the rates of the two reactions in parellel. Second, the fully inhibited state is attained relatively slowly on the usual time scale of enzyme assays. The syncatalytic nature of the inhibition indicates that the state in which the enzyme binds fluoride appears at an intermediary step of the pyrophosphatase reaction. It seems unlikely that the low rate of the attainment of the fully inhibited state should be due to slow addition of fluoride to the enzyme. Rather, it means that the concentration of the enzyme in the state in which binding occurs is very low or that the binding is accompanied by slow conformational changes of the protein molecule.

Two possible mechanisms of the fluoride effect on the pyrophosphatase may be suggested. The binding of F^- may occur at the active site of the enzyme. As a result, subsequent addition of the substrate will cause steric hindrance of the release of the inhibitor and therefore enhnace its binding. Implicit in this mechanism is the assumption that the bound F^- anion blocks only the cleavage of the substrate and does not interfere markedly with its binding. Such arrangement of the substrate and inhibitor at the active site seems quite feasible because of the very small size of the fluoride ion. Alternatively, the substrate might cause exposure of an additional site on the enzyme through a conformational change of the enzyme molecule occuring at some step of the catalytic re-

action. In this case, the inhibition of the overall reaction may be due to stabilization of one of the conformations of the protein molecule.

A similar stabilizing effect of the substrate phenyl butirate of the F^--carboxylesterase complex has been reported by Haugen and Suttie [25]. The binding of the fluoride-phosphate pair to enolase [26–28] and succinate dehydrogenase [29] is also characterized by positive cooperativity.

The interaction of fluoride with pyrophosphatase as well as with several other proteins [25,30–35] becomes stronger at low pH values. The assignment of the ionization site either to the protein or to the inhibitor proved to be difficult, since the corresponding pK_a is small. The results obtained with several proteins for which measurements can be performed at high H^+ concentration show [32,34,35] that the enhancement of inhibition in the acidic pH region is due to protonation of a protein group rather than of the fluoride ion (pK_a for HF is equal to 3.2, Ref. 36). If this is also valid for pyrophosphatase, the pK_a of the enzyme group controlling k_i must be less than 5.5 and this group may be a carboxyl of imidazole residue.

The nature of the enzyme groups forming the fluoride-binding site in pyrophosphatase is unknown. The magnesium ions linked to the protein molecule are, apparently, the most likely candidates. Indeed, the requirement for metals is so common a property among the enzymes whose activity is affected by fluoride, that the sensitivity to this anion was suggested as a test for the participation of metals in their functioning [1]. Subsequent studies showed, however, that some of the enzymes strongly inhibited by fluoride, (esterases for example [37]), have no requirement for metals. It is possible that the atoms forming the immediate environment of this inhibitor may vary in different proteins.

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