Fluoride is a strong and specific inhibitor of (*asymmetrical*) Ap_4A hydrolases

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Fluoride acts as a noncompetitive, strong inhibitor of (*asymmetrical*) Ap_4A hydrolases (EC 3.6.1.17). The K_i values estimated for the enzymes isolated from seeds of some higher plants (yellow lupin, sunflower and marrow) are in the range of 2–3 μM and I_{50} for the hydrolase from a mammalian tissue (beef liver) is 20 μM. The anion, up to 25 mM, does not affect the following other enzymes which are able to degrade the bis(5′-nucleosidyl)-oligophosphates: *Escherichia coli* (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41), yeast Ap_4A phosphorylase (EC 2.7.7.53), yellow lupin Ap_3A hydrolase (EC 3.6.1.29) and phosphodiesterase (EC 3.1.4.1). None of halogenic anions but fluoride affects the activity of (*asymmetrical*) Ap_4A hydrolases. Usefulness of the fluoride effect for the in vivo studies on the Ap_4A metabolism is shortly discussed.

Fluoride; Enzyme inhibition; Ap_4A hydrolase

1. INTRODUCTION

Cellular levels of bis(5′-nucleosidyl)oligophosphates such as Ap_4A, Ap_3A or Ap_4G are probably modulated both by the synthesizing and degrading enzymes. Till now, to the former ones belong some aminoacyl-tRNA synthetases [1–5] and Ap_4A phosphorylase [6–8] and the degrading enzymes are: (i) specific enzymes: (*asymmetrical*) Ap_4A hydrolase (EC 3.6.1.17) occurring in higher eucaryotes [9–12]; (*symmetrical*) Ap_4A hydrolase (EC 3.6.1.41) which had been found in slime mold *Physarum polycephalum* [13] and in different bacteria [14]; Ap_4A phosphorylase (EC 2.7.7.53) found first in yeast [6] and then in *Euglena gracilis* [15], eucaryotic [11,16] and procaryotic [17] Ap_3A hydrolases (EC 3.6.1.29); and (ii) the nonspecific hydrolases: phosphodiesterase type I (EC 3.1.4.1) [11,18–21], nucleotide pyrophosphatase (EC 3.6.1.9) [21,22] and, reported very recently, 5′-nucleotidase (EC 3.1.3.5) from *E. coli* [23]. Among ions affecting activities of the enzymes, the divalent metal cations are the best recognized. Most of the enzymes require the cations, mainly Mg^{2+}, as cofactors. The (*symmetrical*) Ap_4A hydrolase from bacteria needs Co^{2+} for optimum activity and is practically inactive in the presence of Mg^{2+} [14]. Zn^{2+} stimulates dramatically the synthesis of the dinucleotides catalyzed by lysyl-, phenylalanyl-, and alanyl-tRNA synthetases [2–5] and inhibits the rat [24] and lupin [11] Ap_3A hydrolases as well as the rat liver Ap_4A hydrolase [25]. In turn, Ca^{2+} was demonstrated to inhibit the (*asymmetrical*) Ap_4A hydrolases from rat liver [26], yellow lupin seeds [11] and *Artemia* [12] and strongly stimulate the phosphodiesterase I from rat liver [19] and the nucleotide pyrophosphatase and phosphodiesterase I from *Ph. polycephalum* [21].

This report describes a strong and selective inhibitory effect exerted by the smallest anion, fluoride, on the (*asymmetrical*) Ap_4A hydrolases from different organisms. Since fluoride appeared to be inactive on other enzymes degrading bis(5′-nucleosidyl)oligophosphates, it can become a precise tool which can help to understand the role of those dinucleotides and the specific Ap_4A hydrolases at least in eucaryotic cells.

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Abbreviations: Ap_4A, P^1,P^4-bis(5′-adenosyl)tetraphosphate; Ap_3A, P^1,P^3-bis(5′-adenosyl)triphosphate; Ap_4G, adenosine 5′-P^1-tetraphospho-P^4-5′′′-guanosine; Ap_5A, P^1,P^5-bis(5-′adenosyl)pentaphosphate

2. MATERIALS AND METHODS

2.1. *Chemicals*

Salts and sheets for thin-layer chromatography were from Merck and unlabeled nucleotides from Sigma. The phosphonate analogue of Ap_4A, β,β'-methylene-Ap_4A, was synthesized by Dr N.B. Tarussova, from the Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow. Di[2,8-^{3}H]adenosine tetraphosphate (8.7 Ci/mmol) and [2,5,8-^{3}H]adenosine 5′-triphosphate (45 Ci/mmol) were from Amersham. The radioactive Ap_3A was synthesized enzymatically and purified as described earlier [11].

2.2. *Enzymes*

Yellow lupin seed Ap_3A hydrolase, (*asymmetrical*) Ap_4A hydrolase and phosphodiesterase [11], *E. coli* (*symmetrical*) Ap_4A hydrolase [14] and yeast Ap_4A phosphorylase [6] were obtained as described

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previously. The (*asymmetrical*) Ap_4A hydrolases from sunflower and marrow seeds as well as the one from beef liver were purified by ammonium sulfate precipitation (35–60% saturation) and gel filtration on Sephadex G-75 superfine column (1.14 × 120 cm) equilibrated with 50 mM potassium phosphate (pH 6.8) containing 10% glycerol and 1 mM 2-mercaptoethanol. The preparations from plants contained traces of activity hydrolyzing ATP and ADP.

2.3. *Enzyme assays*

Initial velocities of the reactions catalyzed by the investigated enzymes were estimated as before [6,11,14] at varying concentrations of NaF. Radioactive substrates, Ap_4A or Ap_3A, were separated by thin-layer chromatography from the products, ATP and AMP or ADP. Spots of AMP (in case of (*asymmetrical*) Ap_4A hydrolases, Ap_3A hydrolase and phosphodiesterase) and of ADP (in case of (*symmetrical*) Ap_4A hydrolase and Ap_4A phosphorylase) were cut out from the chromatograms and radioactivity counted. Assaying of the activity of (*asymmetrical*) Ap_4A hydrolase is described in details in the legend to fig.1.

3. RESULTS AND DISCUSSION

3.1. *How the inhibition of (*asymmetrical*) Ap_4A hydrolase by fluoride was noticed*

Analyzing incubation mixtures in which Ap_4A had been hydrolyzed by partially purified Ap_4A hydrolase from lupin seeds I noticed that ATP, one of the reaction products, was slowly degraded to ADP and AMP. The same degradation was observed in the mixtures containing only ATP or ADP which indicated that the Ap_4A hydrolase fraction is contaminated by some ATPase and/or apyrase. Trying to inhibit those activities I used fluoride (20 mM NaF or KF), the known inhibitor of various ATPases and phosphatases [27–29]. I found that, in fact, the anion did inhibit both the plant phosphatase activities and the Ap_4A hydrolase. Since the inhibitory effect of fluoride on the enzymes involved in the metabolism of Ap_4A or Ap_3A had not been reported, I found it worthy to explore that finding. Unless otherwise indicated, the characterization of the inhibitory effect was performed on homogeneous preparation of the (*asymmetrical*) Ap_4A hydrolase from lupin seeds [11].

3.2. *Are (*asymmetrical*) Ap_4A hydrolases from other eucaryotic organisms also inhibited by fluoride?*

Before this question could be solved, we purified partially (*asymmetrical*) Ap_4A hydrolases from seeds of two other plant species, sunflower and marrow, and from an animal tissue, beef liver (see section 2). Using the routine assay I proved that all the hydrolases were strongly inhibited by NaF (for more details see below). It can be added that the inhibition was also observed when Ap_4A analogues (Ap_5A, Gp_4G and β,β'-methylene-Ap_4A), were used as substrates.

3.3. *Do other halogenic anions affect the lupin (*asymmetrical*) Ap_4A hydrolase?*

Sodium or potassium chloride, bromide and iodide, at 20 mM final concentration, were tested with the lupin hydrolase. It was demonstrated that none of the anions either stimulated or inhibited the enzyme activity. Also the following compounds (up to 20 mM), KCN, KCNS, NaN_3 and potassium phosphate (pH 8.0), had no effect on the hydrolase activity.

3.4. *Does fluoride also inhibit other enzymes catalyzing degradation of Ap_4A and/or Ap_3A?*

Fluoride (20 mM) does not affect the following enzymes: (*symmetrical*) Ap_4A hydrolase from *E. coli*, Ap_4A phosphorylase from yeast, Ap_3A hydrolase and phosphodiesterase from yellow lupin seeds.

3.5. *Reversibility and time dependence of the inhibition*

In order to check whether fluoride acts as a reversible or irreversible inhibitor of the lupin (*asymmetrical*) Ap_4A hydrolase, we preincubated 100 μl of the enzyme stock solution with 20 mM NaF for 15 min at room temperature and then passed it through a small (0.5 × 5 cm) Sephadex G-50 fine column to separate protein from NaF. Samples without NaF were run as a control. As the preincubated and desalted sample appeared to be fully active, fluoride seems to be a reversible inhibitor of the hydrolase. The inhibition does not depend upon time as shown by the following experiment: the stock solution of the lupin Ap_4A hydrolase was diluted twice with 200 μM NaF and kept on ice. After 1, 30, 60 and 120 min, the 2-μl aliquots were diluted 6-fold with 25 mM Hepes/KOH buffer, pH 8.0, and 10-μl portions were subsequently used to estimate the velocity of Ap_4A hydrolysis in the presence of about 3.3 μM NaF (final concentration). The velocities did not differ.

3.6. *Estimation of the K_i and I_{50} for fluoride in different (*asymmetrical*) Ap_4A hydrolase systems*

Two series of experiments were carried out to estimate the K_i and I_{50} values for the lupin Ap_4A hydrolase. To determine K_i, we measured the initial velocities at various Ap_4A concentrations (3–25 μM) in the absence and presence of 2 and 6 μM NaF. In this system, the K_m for Ap_4A is 1 μM [11]. Parallel kinetic lines (not shown) obtained in the Eadie-Hofstee plotting (v vs v/S) indicated that the NaF is a noncompetitive inhibitor of the Ap_4A hydrolase. The computed K_i value for the Ap_4A hydrolase was 2 μM. In other experiments, the I_{50} values were determined at fixed 500 μM Ap_4A and varying concentrations of NaF (fig.1). The I_{50} values for the Ap_4A hydrolases from yellow lupin, sunflower and marrow seeds were 2–3 μM and that for the beef liver enzyme was 20 μM. In the case of the plant enzymes, our measurements confirmed the relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction ($K_i = I_{50}$), applying under noncompetitive or un-

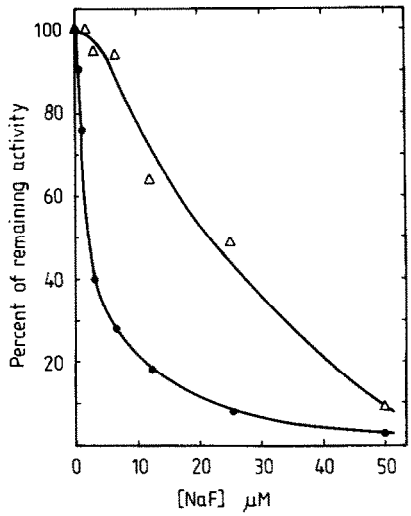

Fig.1. Inhibition of (*asymmetrical*) Ap_4A hydrolases by NaF. The incubation mixtures (50 μl final volume) contained 50 mM Hepes/KOH buffer, pH 8.0, 5 mM $MgCl_2$, 20 μM dithiothreitol, 500 μM [^{3}H]Ap_4A (about 250000 cpm), varying concentrations of NaF and rate-limiting amounts of yellow lupin (●) or beef liver (△) Ap_4A hydrolase. Incubation was carried out at 30°C. Initial velocities of Ap_4A hydrolysis were estimated from 4 experimental points of the time-course analysis. At time intervals (usually after 5, 10, 15 and 20 min), the 5-μl aliquots were transferred onto poly(ethylenimine)-cellulose plastic sheets (from E. Merck), the standard AMP was added, and the chromatogram developed for 20 min in 75% methanol followed by 1 h in 0.85 M LiCl. (In the chromatographic system the R_f values for Ap_4A, AMP, ADP and ATP are 0.17, 0.39, 0.24 and 0.07, respectively.) Spots of AMP visualized under UV light were cut out and their radioactivity was determined by liquid scintillation counting.

competitive kinetics [30]. The inhibition pattern of the beef liver Ap_4A hydrolase is not Michaelian. The sigmoidal NaF-dependence observed with this enzyme (fig.1) suggests that more than a single fluoride ion is involved in the interaction with the enzyme molecule.

3.7. *General considerations*

The fluoride K_i (I_{50}) values found for the (*asymmetrical*) Ap_4A hydrolases from higher plants are, to the best of my knowledge, the lowest ones among those reported for that anion in various enzymatic systems which are usually in the millimolar range. The lowest value I could find (I_{50} = 30 μM) concerns the bacterial pterin carboxylic acid deaminase [31]. The I_{50} values reported for some enzymes metabolizing nucleotides, the nucleotide pyrophosphatase from potatoes [32] and 5′-AMP deaminase from rabbit muscle [33] are 100 and 5 mM, respectively. The property of fluoride described here raises the possibility of using in vivo the anion as a selective inhibitor of Ap_4A hydrolase without affecting practically other fluoride-sensitive enzymes. Thus the novel effect exerted by fluoride on eucaryotic Ap_4A hydrolases is not only just another example joining the rather long list of enzymes which are inhibited by that anion (for review containing the list of over 70 such enzymes, see [29]). It seems plausible that due to its small ionic radius (0.07 Å, according to 'The Table of Periodic Properties of the Elements' published by Sargent-Welch Scientific Co.), fluoride can easily penetrate cellular structures and reach the Ap_4A hydrolases. Another interesting problem emerging from this study which would be worthy of investigation is the structure of a unique and apparently conservative region of the (*asymmetrical*) Ap_4A hydrolases which interacts so strongly with fluoride.

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