The interaction of phospholipid bilayers with pig heart AMP deaminase: Fourier-transform infrared spectroscopic and kinetic studies

Fabio TANFANI,*‡ Ewa KOSSOWSKA,† Jadwiga PURZYCKA-PREIS,† Mariusz M. ZYDOWO,† Michal WOZNAK,† Elena TARTAGLINI† and Enrico BERTOLI*

*Istituto di Biochimica, Facoltà di Medicina, Università di Ancona, Via Ranieri, 60100 Ancona, Italy, and †Department of Biochemistry, Medical Academic School, Gdansk 80-210, Debinki 1, Poland

The interaction of pig heart AMP deaminase with different chemical species of phosphatidylcholine and with natural plasma membranes has been investigated. Phospholipids added to the system either as natural biological membranes (plasma membrane vesicles) or in the form of liposomes containing unsaturated phosphatidylcholine considerably enhanced AMP deaminase activity. The secondary structure of pig heart AMP deaminase in the absence and in the presence of dioleoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine liposomes was investigated

by Fourier-transform infrared spectroscopy. Quantitative analysis of the amide I band showed that the enzyme contains 45 % β -sheets, 28 % α -helix, 16 % turns and 11 % non-ordered structure. In the presence of dioleoyl phosphatidylcholine liposomes, the β/α content ratio decreased; this decrease was dependent on the amount of lipid added. This phenomenon was not observed in the case of dipalmitoyl phosphatidylcholine liposomes. These data suggest a possible role for membrane phospholipids in the regulation of AMP deaminase activity.

INTRODUCTION

AMP deaminase (AMP aminohydrolase; EC 3.5.4.6) catalyses the hydrolytic deamination of AMP to IMP. This ammonia-forming enzyme of the purine nucleotide cycle occurs in the cytosol of most eukaryotic cells, including those of mammals, bird, amphibians [1], plants [2] and yeast [3]. In 1967, Burger and Lowenstein [4] found from their experiments with heart extracts that the presence of ATP may cause a 10-fold decrease in adenosine production from AMP and a 5-fold increase in AMP deamination. Hence the precise regulation of heart AMP deaminase activity as a possible modulator of cellular adenosine levels is of great physiological importance and commands growing attention. Surprisingly, isolated skeletal muscle AMP deaminase was found to be sensitive to the ATP-activating effect at very low, non-physiological, concentrations of K^+ only [5,6]. A search for ATP-activated AMP deaminase in the heart led us to isolate in 1978 a substrate-specific pig heart AMP deaminase, which is activated by ATP even in the presence of a high K^+ concentration (150 mM) [7]. The ATP-activated pig heart AMP deaminase was unexpectedly sensitive to phospholipids. Phosphatidylcholine (PC)-containing liposomes were found to activate this enzyme in the presence of ATP [7]. On the other hand, phosphatidate bilayers composed of dioleoyl phosphatidate were found to exert non-competitive inhibition on the AMP deaminase with a K_i of 15 μ M, three orders of magnitude lower than that for orthophosphate, a well-known negative allosteric effector of AMP deaminase [8]. Another interesting finding was the established dependence of the inhibition by phosphatidate 'effectors' on membrane fluidity. Phosphatidate species containing saturated fatty acids were either non-inhibitory or inhibited enzyme activity rather poorly [8]. Nevertheless, fluidization of the saturated phosphatidate species by alkalization of the medium resulted in the appearance of an

inhibitory effect of dilauroyl phosphatidate bilayers on pig heart AMP deaminase [8]. An activatory effect of PC bilayers on pig heart AMP deaminase described previously [7] involved natural egg yolk PC. Naturally occurring PC species contain mixtures of saturated and unsaturated fatty acids, with an average number of *cis* double bonds of 1.1–1.5; in the structurally related sphingomyelin, the corresponding number is only 0.1–0.35 [9]. This difference was taken into consideration when investigating the effect of PC on pig brain AMP deaminase [10]. The different effects of natural PC, which was activatory, and sphingomyelin, which was inhibitory towards the pig brain enzyme, were found to be dependent on the differences between the interfacial regions of these two phospholipid molecules, as dipalmitoyl PC (DPPC) was not inhibitory [10].

The interaction of heart AMP deaminase with PC species containing different fatty acids has not been investigated so far. In the last decade, detailed studies on protein secondary structure and lipid–protein interactions have been made possible by the application of Fourier-transform infrared (F.t.i.r.) spectroscopy. In this paper, investigations on the interaction of pig heart AMP deaminase with different chemical species of PC and with natural plasma membranes are presented. Changes in the kinetic properties of the enzyme in the presence of phospholipids have been shown to be accompanied by changes in the i.r. spectrum of the protein.

MATERIALS AND METHODS

Materials

Bistris, adenosine 5'-[β - γ -imido]triphosphate (p[NH]ppA), ATP, dimyristoyl PC (DMPC), DPPC and dioleoyl PC (DOPC) were purchased from Sigma. Cellulose phosphate P-11 was obtained from Whatman, Maidstone, Kent, U.K. Deuterium oxide (99.9 % 2H) was purchased from Aldrich.

Abbreviations used: F.t.i.r., Fourier-transform infrared; PC, phosphatidylcholine; DOPC, dioleoyl PC; DPPC, dipalmitoyl PC; DMPC, dimyristoyl PC; p[NH]ppA, adenosine 5'-[β - γ -imido]triphosphate; $S_{0.5}$, half-saturating substrate concentration.

‡ To whom correspondence should be addressed.

Enzyme preparation

AMP deaminase from pig heart was prepared and purified by using cellulose phosphate chromatography as described by Purzycka-Preis and co-workers [8].

Preparation of liposomes and plasma membranes

Small unilamellar liposomes containing different kinds of phospholipids were prepared by sonication under an atmosphere of argon and at a temperature above that of the main lipid phase transition, as described previously [8]. The resulting lipid dispersions were freshly prepared every day. Plasma membranes from rat heart were prepared using the hypotonic swelling/lithium bromide extraction method [11].

Enzyme assay

The incubation mixture (final volume 0.5 ml) contained 100 mM potassium succinate, pH 6.5, 100 mM KCl, 10 μ g of enzyme protein, AMP and other additions as indicated in the Figures. The reaction was carried out for 10 min at 30 °C or 45 °C after a preincubation for 20 min at the same temperature. It was started by the addition of the enzyme solution and terminated by the addition of phenol reagent; the amount of ammonia liberated was then determined. To investigate the effects of liposomes, typically 50 μ l of enzyme solution containing 10 μ g of protein was preincubated at 30 °C for 20 min with 250 μ l of liposome suspension containing the amount of phospholipids indicated in the Figures. In the case of DPPC liposomes, the enzyme solution was preincubated with the lipid suspension for 20 min at 30 °C or 45 °C and the reaction was carried out at the same temperature. The same procedure was used in experiments with heart plasma membranes, with preincubation and reaction being carried out at 30 °C. To avoid the interference of sarcoplasmic membrane ATPase activities in these particular experiments ATP was replaced by the non-hydrolysable ATP analogue p[NH]ppA.

Sample preparation for F.t.i.r. experiments

The protein solution (3 %, w/v) used for F.t.i.r. measurements was obtained in 10 mM Bistris/100 mM KCl by concentrating the purified AMP deaminase in Centricon-30 tubes (Amicon Division, W.R. Grace, Danvers, MA, U.S.A.) and washing several times with buffer prepared in either H₂O or ²H₂O (pH or p²H 6.5). For the experiments performed in ²H₂O, the p²H (i.e. pH meter reading + 0.4) [12] was adjusted to 6.5 with NaO²H/²HCl. Before collecting i.r. spectra, the protein prepared in ²H₂O was maintained for 2 days at room temperature.

For the experiments on protein-lipid interactions, the appropriate amount of dried DOPC or DPPC was hydrated at room temperature or 45 °C respectively with the concentrated protein solution. The samples were vortex-mixed several times to ensure complete dispersion of the lipid. Typically, 30 μ l of protein solution was added to 3 or 6 mg of dried lipid. Before recording the spectra, these samples were kept for at least 30 min at 30 °C or 45 °C. In all experiments performed in the presence of ATP, the nucleotide was added to a final concentration of 2 mM.

I.r. spectra

A total of 512 scans were collected for each sample in a Perkin-Elmer 1760 F.t.i.r. spectrometer. Data were encoded every 1 cm⁻¹ at 2 cm⁻¹ resolution with a triangular apodization function. Samples were placed in a Specac 20500 cell (Specac

Ltd., Orpington, Kent, U.K.) fitted with CaF₂ windows and 12 μ m or 6 μ m tin spacers. During data acquisition, the spectrometer was continuously purged with dry air at a dew point of -40 °C. Buffer spectra were recorded under the same temperature and scanning conditions as sample spectra. Subtraction of ²H₂O spectrum was adjusted to the removal of the ²H-O-²H bending absorption near 1220 cm⁻¹ [13]. Second derivative spectra were calculated over a 13-data-point range (13 cm⁻¹). Spectral deconvolution was performed using the Perkin-Elmer ENHANCE function, which is analogous to the method developed by Kauppinen et al. [14]. Deconvolution parameters were 20 for the half-width at half-height and 2.5 for the resolution enhancement factor.

Curve fitting

The deconvoluted amide I band contour was fitted with Gaussian/Lorentzian curves. The calculations were performed according to the method reported by Blume et al. [15]. The position and the number of amide I components, used as input for the curve-fitting program, were obtained from the second derivative, fourth derivative and deconvoluted absorption spectra. In each Gaussian/Lorentzian curve the position, width and height were considered to be free parameters, whose values were fitted by a recursive method. The position of the curves was allowed to vary within a range of ± 5 cm⁻¹ with respect to the frequencies used as input. The goodness of fit is visualized by a line obtained as the difference between the experimental values and the calculated ones, and by the corresponding S.D.

Analytical procedures

Ammonia was measured by the phenol/hypochlorite method described by Chaney and Marbach [16]. Protein concentration was determined either by the method of Lowry et al. [17] or by the method of Warburg and Christian as described by Layne [18]. Phospholipid phosphorus was determined as described by Chen et al. [19].

RESULTS

The activity of AMP deaminase isolated from pig heart muscle was found to be influenced by both ATP and p[NH]ppA (Figures 1a and 1b). To avoid interference by sarcoplasmic membrane ATPase activities, ATP in this particular experiment was replaced by the non-hydrolysable analogue p[NH]ppA. In the absence of ATP or p[NH]ppA, the addition of liposomes or plasma membrane vesicles did not influence the enzyme kinetics. Addition of ATP or p[NH]ppA to the reaction mixtures shifted the kinetics from a sigmoidal to a hyperbolic pattern, thereby lowering the apparent *S*_{0.5} (half-saturating substrate concentration) for AMP. This value changed from 5 mM to 1.5 mM after the addition of ATP. p[NH]ppA exerted a lesser activatory effect, changing the *S*_{0.5} from 5 mM to 2.4 mM (Figure 1b). In the presence of ATP, addition of liposomes resulted in a further shift in the *S*_{0.5} to 0.4 mM and an increase in the *V*_{max} of about 1.66-fold (66 %) (Figure 1a). Addition of plasma membrane vesicles in the presence of p[NH]ppA caused a shift in the *S*_{0.5} of AMP deaminase to 0.9 mM and an increase in the *V*_{max} of about 1.45-fold (45 %) (Figure 1b). Therefore, phospholipids added to the system either within a natural biological membrane (plasma membrane vesicles) or in the form of liposomes exerted a considerable activatory effect on AMP deaminase activity. The effect of liposomes was found to increase in a saturating fashion on increasing the amount of DOPC (Figure 2). As little as

Figure 1 Effect of DOPC (a) and heart plasma membrane vesicles (b) on ATP-activated and p[NH]ppA-activated AMP deaminase activity

Activity was measured under the following conditions: ●, in the absence of ATP, p[NH]ppA and membranes; ○, in the presence of membranes; △, □, in the presence of 1 mM ATP and p[NH]ppA respectively; ▲, in the presence of 1 mM ATP and 375 nmol of phospholipid; ■, in the presence of 1 mM p[NH]ppA and plasma membrane vesicles equivalent to 170 nmol of phospholipid phosphorus. For experimental details, see the text.

Figure 2 Influence of increasing amounts of DOPC on the activity of 1 mM ATP-activated AMP deaminase from pig heart (□), and substrate saturation curve of AMP deaminase in the presence of 1 mM ATP (○)

For experimental details, see the text.

50 nmol of added phospholipid exerted a considerable effect on ATP-activated AMP deaminase activity. However, oleoyl isop-PC, which forms a non-bilayer micellar structure, was without effect on AMP deaminase activity (results not shown). The

Figure 3 Effect of DOPC- (○), DMPC- (●) and DPPC- (△) containing liposomes on ATP-activated AMP deaminase from pig heart

Incubations were carried out at 30 °C for 20 min. In the case of DPPC liposomes, the incubation and the reaction were carried out at both 30 °C (curve A) and 45 °C (curve B). Other experimental details were described in the Materials and methods section.

Figure 4 I.r. spectra (1800–1600 cm^{-1} region) of AMP deaminase in $^2\text{H}_2\text{O}$ at 30 °C after subtraction of the buffer spectrum

Curve a, original absorbance spectrum; b, same spectrum after band narrowing by Fourier deconvolution; c, same spectrum after resolution enhancement by second derivation.

activatory effect of the bilayer was shown to be dependent on the viscosity factor of the membrane (Figure 3). DPPC-containing liposomes were without effect on AMP deaminase activity, in contrast with DOPC-containing vesicles, whereas DMPC-containing liposomes were found to exert a lower activatory effect (Figure 3). AMP deaminase preincubated at 45 °C for 20 min (reaction also at 45 °C) with DPPC liposomes in the presence of ATP showed about 20 % lower activity than that preincubated at 30 °C (reaction also at 30 °C) (Figure 3).

The original absorbance spectrum (amide I) of AMP deaminase at 30 °C, after subtraction of the buffer spectrum, is shown in Figure 4 (curve a). The absorbance maximum is located at 1641 cm^{-1} , which is lower than that found in water. This effect

Figure 5 Deconvoluted amide I band contour of AMP deaminase with the best-fit Gaussian/Lorentzian individual component bands for AMP deaminase at 30 °C

A weighting factor of 0.8 was used for the Gaussian function. The symbols α , β , t and r stand for α -helices, β -structures, turns and random (non-ordered) structures respectively; aa stands for amino acid side-chain absorption. The line (d) visualizes the goodness of fit and represents the difference between the experimental values and the calculated ones. The S.D. was 0.0065.

is due to the exchange of amide protons with ^2H [20,21]. Another effect resulting from the $^2\text{H}_2\text{O}$ medium is the decrease in the intensity of the amide II band, as upon $\text{H}/^2\text{H}$ exchange this band is shifted at about 1450 cm^{-1} [20,21]. Before collecting the i.r. spectra, the protein was stored at room temperature in $^2\text{H}_2\text{O}$ medium for 2 days; after this time no further changes in the amide II intensity were observed, suggesting that no further $\text{H}/^2\text{H}$ exchange occurred. The second derivative and deconvoluted spectra of AMP deaminase (Figure 4) revealed amide I component bands at 1687, 1675, 1667, 1653, 1643, 1635, 1627 and 1610 cm^{-1} . In water (results not shown), these bands were found at 1690, 1678, 1675, 1657, 1653, 1634 and 1614 cm^{-1} . According to well-defined criteria [22] we assigned the band observed in $^2\text{H}_2\text{O}$ medium at 1653 cm^{-1} to α -helix, the bands at 1635 and 1627 cm^{-1} to β -sheet structure, the bands at 1687 and 1667 cm^{-1} to β -turns, and the band at 1643 cm^{-1} to unordered structure. The band observed at 1675 cm^{-1} , which was also present in the spectrum recorded in water-based medium, is most likely due to β -sheet structure, and the band at 1610 cm^{-1} probably represents amino acid side-chain absorption [23].

Figure 5 shows the curve-fitted amide I band contour of AMP deaminase in $^2\text{H}_2\text{O}$ at 30 °C. The best fit was obtained with a

Figure 6 I.r. spectra (1800–1600 cm^{-1} region) of AMP deaminase in the presence of 2 mM ATP and DOPC or DPPC liposomes at 30 °C

Curve a, amide I band of AMP deaminase in the absence of lipids (maximum at 1641 cm^{-1}); b, i.r. spectrum in the presence of 3 mg of DOPC (maximum of the amide I band at 1645 cm^{-1}); c, i.r. spectrum in the presence of 6 mg of DOPC (maximum of the amide I band at 1653 cm^{-1}); d, i.r. spectrum in the presence of 6 mg of DPPC (maximum of the amide I band at 1641 cm^{-1}).

Gaussian weighting factor of 0.8. The calculations show that the native enzyme contains 28% α -helix, 45% β -sheet structure, 11% unordered structure and 16% turns (see Table 1). In the presence of ATP, the secondary structure of the enzyme did not change significantly. This is shown by the identical positions and intensities of the amide I component bands and by the quantitative estimation of the secondary structure of the enzyme in the presence of ATP (Table 1). However, in the presence of ATP plus 3 mg of DOPC, the amide I band contour of the protein changed significantly and the maximum was shifted from 1641 to 1645 cm^{-1} . If the amount of DOPC was doubled, the maximum of the amide I band was further shifted to 1653 cm^{-1} . These data are shown in Figure 6, in which the spectrum of AMP deaminase at 30 °C in the presence of DPPC (6 mg) and 2 mM ATP, and the spectrum of AMP deaminase at 30 °C in the absence of lipids (as a comparison), are also reported. The small peak at 1602 cm^{-1} observed in the presence of DOPC is due to the double bond of the lipid; all spectra were normalized at the carbonyl stretching band of lipids. As Figure 6 shows, the addition of DPPC to the enzyme solution did not cause remarkable changes in the amide

Table 1 Estimation of AMP deaminase secondary structure

The shape of the deconvoluted amide I band of AMP deaminase was simulated by Gaussian/Lorentzian functions. The best fit was obtained with a Gaussian weighting factor of 0.8.

	Content (%)					
	30 °C, no ATP, no lipids	30 °C, ATP, no lipids	30 °C, ATP, 3 mg of DOPC	30 °C, ATP, 6 mg of DOPC	30 °C, ATP, 6 mg of DPPC	45 °C, ATP, 6 mg of DPPC
β -Structures	45	46	39	29	46	44
α -Helices	28	31	37	43	34	30
Random	11	9	12	8	9	14
Turns	16	14	12	20	11	12
β/α ratio	1.60	1.48	1.05	0.67	1.35	1.46

Figure 7 Deconvoluted and second derivative spectra of AMP deaminase in the presence of 2 mM ATP and DOPC or DPPC liposomes

Bottom, deconvoluted spectra; top, second derivative spectra. (a) I.r. spectra in the presence of 3 mg of DOPC at 30 °C; (b) i.r. spectra in the presence of 6 mg of DOPC at 30 °C; (c) i.r. spectra in the presence of 6 mg of DPPC at 30 °C (A) and 45 °C (B).

I band, in contrast with DOPC. These changes in the amide I band contour could be postulated to be due to the closeness of the strong C=O stretching band of the lipids. However, this seems not to be the case. In fact, since the DPPC/protein and DOPC/protein weight ratios were the same (as also shown by the C=O/amide I band intensity ratios in Figure 6), any possible contribution of the C=O stretching band of lipids to the amide I band should be the same, and consequently the amide I band contour should be affected in the same way in both cases. Hence the different amide I band contour observed in the presence of DOPC is most likely due to changes in the secondary structure of the protein induced by this lipid. The second derivative and deconvoluted spectra of the protein in the presence of DOPC and DPPC are shown in Figure 7. The changes in the amide I band contour induced by DOPC are clearly observed here. In particular, in the presence of 3 mg of DOPC (Figure 7a) the band at 1636 cm⁻¹ (due to β -sheets) decreased in intensity compared with that found in the absence of lipids. When the amount of DOPC was doubled (Figure 7b) the intensity of the 1636 cm⁻¹ band decreased further. These effects were not observed in the presence of 6 mg of DPPC (Figure 7c), since the spectrum of the protein recorded at both 30 and 45 °C resembled that of the enzyme in the absence of lipids, as shown by the band due to the β -sheet structure which remained greater in intensity than that due to α -helix. The quantitative analysis of the amide I band of AMP deaminase in the absence and in the presence of DOPC and DPPC confirms that DOPC induces changes in the secondary structure of the enzyme while DPPC does not (Table 1).

DISCUSSION

In spite of the extensive discussion in the literature on the regulation of adenosine production and adenylate metabolism, the process is only poorly understood. The results obtained by Newby [24] indicated that soluble 5'-nucleotidase from pigeon heart, believed to regulate adenosine production, may be associated via a weak ionic interaction with an organelle present in the low-speed particulate fraction. We observed that AMP

deaminase is also able to associate weakly with some intracellular organelles (results not shown). Activation of AMP deaminase by natural sarcolemmal membrane vesicles seems to be another regulatory factor controlling the size of the AMP pool for adenosine production. Experiments performed using heart extracts [4] revealed that ATP may cause a 5-fold increase in AMP deamination and a 10-fold decrease in adenosine production from AMP. On the other hand, it has been shown that orthophosphate exhibits a negative allosteric effect on AMP deaminase. Egg yolk PC liposomes did not influence this inhibitory effect of orthophosphate [8]. High activity of the enzyme observed in the presence of ATP and PC-containing membranes may thus occur in the tissue when intramitochondrial ATP production exceeds its hydrolysis. In this particular situation, adenosine, as a factor necessary for increasing the oxygen supply through an increased coronary blood flow, is not critically required, and thus the AMP concentration should be low. ATP, an activator of AMP deaminase, is also required for full lipid-dependent activation (Figure 1a), but may undergo excessive degradation in certain metabolic situations (e.g. ischaemia). If excessive orthophosphate production and ATP depletion take place, AMP deamination is inhibited and enhanced adenosine production may occur. Kinetic data obtained with AMP deaminase showed that the enzyme is activated by ATP. This activating effect was further increased by the presence of egg PC and DOPC, but not by DPPC liposomes at either 30 or 45 °C. Since no remarkable differences in AMP deaminase secondary structure were observed in the presence of the nucleotide, the activating effect of ATP might be due to changes in the conformation of the enzyme (i.e. tertiary structure) or to small changes in its secondary structure which cannot be detected by i.r. spectroscopy. The interaction of AMP deaminase with DOPC liposomes could be explained by assuming that the enzyme possesses hydrophobic segment(s) which might be able to interact with the lipid acyl chains. These interactions would occur if the membrane possesses suitable fluidity, as suggested by both the kinetic and i.r. data. The fact that AMP deaminase preincubated at 45 °C for 20 min in the presence of ATP showed approx. 20% lower activity than that at 30 °C, both in the absence and in the presence of DPPC, suggests that at this temperature the enzyme undergoes changes in its structure. These changes probably involve the tertiary structure of the enzyme, since only small differences in secondary structure at 30 °C and 45 °C were found (see Table 1).

Regulation of the adenine nucleotide pool by AMP degradation has been demonstrated to occur both in prokaryotes and in eukaryotes. This regulation is proposed to occur via the activity of AMP nucleosidase in prokaryotes [25], whereas in eukaryotes AMP deaminase activity seems to be the regulating factor [26,27]. The enzyme isolated from pig heart displays catalytic and regulatory properties similar to those of AMP deaminases from rat kidney and heart, including allosteric activation by ATP and PC and inhibition by orthophosphate and phosphatidate [7,8]. The catalytic functions of rat muscle AMP deaminase are located near the C-terminal portion of the enzyme [28]. Comparison of the amino acid sequences of the C-terminal portions of rat muscle and yeast AMP deaminases revealed a number of regions with a degree of sequence identity of $\geq 85\%$, including one completely identical sequence of 32 amino acids [28]. These conserved sequences are expected to correspond to the regions in AMP, ATP and orthophosphate binding [28]. In the N-terminal portions of these enzymes, the degree of identity is only 11%. Similarities between the regulatory properties of eukaryotic heart AMP deaminase and of the yeast enzyme (activation by ATP and inhibition by orthophosphate)

has led to the proposal that they have similar metabolic functions. AMP deaminase is the main enzyme degrading AMP in the cell-free extracts of yeast [29]. Similar conclusions have been drawn from experiments performed on extracts of rat heart [30]. The transient ATP-dependent interaction of cytosolic heart AMP deaminase with intracellular membrane lipids demonstrated in this paper may be of importance in the subtle regulation of adenylate degradation in the heart. It is reasonable to postulate that the activation of deaminase by association with membranes at high ATP concentrations may cause a decrease in AMP, the substrate for adenosine production. The opposite may occur when the cell is depleted of ATP. In this case, AMP deaminase would dissociate from the membranes and the AMP concentration would increase, providing an abundance of substrate for adenosine production via the reaction of 5'-nucleotidase.

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