Kinetic characterization of adenosine deaminase activity in zebrafish (Danio rerio) brain

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A B S T R A C T

Adenosine deaminase (ADA; EC 3.5.4.4) activity is responsible for cleaving adenosine to inosine. In this study we described the biochemical properties of adenosine deamination in soluble and membrane fractions of zebrafish (Danio rerio) brain. The optimum pH for ADA activity was in the range of 6.0–7.0 in soluble fraction and reached 5.0 in brain membranes. A decrease of 31.3% on adenosine deamination in membranes was observed in the presence of 5 mM Zn2+, which was prevented by 5 mM EDTA. The apparent Km values for adenosine deamination were 0.22±0.03 and 0.19±0.04 mM for soluble and membrane fractions, respectively. The apparent Vmax value for soluble ADA activity was 12.3±0.73 nmol NH3 min−1 mg−1 of protein whereas Vmax value in brain membranes was 17.5±0.51 nmol NH3 min−1 mg−1 of protein. Adenosine and 2-deoxyadenosine were deaminated in higher rates when compared to guanine nucleosides in both fractions. Furthermore, a significant inhibition on adenosine deamination in both soluble and membrane fractions was observed in the presence of 0.1 mM of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA). The presence of ADA activity in zebrafish brain may be important to regulate the adenosine/inosine levels in the CNS of this species.

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1. Introduction

Adenosine is an important signaling molecule within tissues. This nucleoside can be released depending on the intracellular concentrations, or can be rapidly formed from extracellular ATP breakdown due to ecto-nucleotidase activities (Fredholm, 2002). The effects elicited by adenosine are mediated by metabotropic P1 receptors, which include the negatively coupled to adenylyl cyclase A1 and A3 receptors and the positively coupled to adenylyl cyclase A2A and A2B receptors (Reshkin et al., 2000; Burnstock, 2007).

In the central nervous system (CNS), adenosine acts as a neuromodulator, controlling both inhibitory and excitatory synapses (Burnstock, 2007). Despite the neuroprotective roles promoted by extracellular adenosine, the activation of A2A receptors may aggravate tissue damage (Haskó et al., 2005). Therefore, the interaction of adenosine with its respective purinoreceptors must be tightly regulated. The control of the adenosinergic signaling can be exerted by adenosine uptake via bi-directional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase (ADA; EC 3.5.4.4) (Fredholm et al., 2005). Furthermore, studies have shown that the extracellular concentrations of adenosine may also be regulated by ecto-ADA activity (Franco et al., 1998; Romanowska et al., 2007). A phylogenetic study demonstrated the existence of different ADA-related members, which include ADA1, ADA2, and a similar deduced amino acid sequence named adenosine deaminase like (ADAL) (Maier et al., 2005). These proteins, together with adenosine deaminase (Ade) from yeast and AMP deaminase, are grouped in the adenyl-deaminase family (Maier et al., 2005). Concerning the ADA-related proteins, it has been demonstrated that almost all human ADA activity has been attributed to ADA1 (Zaviyalov and Engstrom, 2005). Despite its intracellular location, ADA1 may act as an ecto-ADA cleaving extracellular adenosine (Franco et al., 1997). This ADA member plays a key role in the immune system, since its activity is important to control the adenosine-mediated inhibition of T-cells proliferation (Gorrell et al., 2001). Moreover, the ADA1 dysfunction in childhood causes the severe combined immunodeficiency (SCID) with severe tissue damages (Mortellaro et al., 2006). The presence of ADA1 in CNS has already been reported (Franco et al., 1997). Studies demonstrated that this enzyme may be colocalized with CD26 (DPP IV-dipeptidyl-peptidase IV), A1, and A2B Receptors in neuronal and non-neuronal cells, suggesting a possible involvement of ecto-ADA to regulate adenosine signaling via P1 receptors (Franco et al., 1998; Ruiz et al., 2000; Herrera et al., 2001).
The other ADA subfamilies comprises ADA2 and ADAL. The deduced amino acid sequence analysis of ADA2 revealed that it is encoded by cat-eye syndrome critical region candidate 1 (CECR1) gene in vertebrates (Zavialov and Engstrom, 2005). It was verified that human plasma ADA2 may be secreted in plasma by activated monocytes (Iwaki-Egawa et al., 2006). Due to its ability to regulate cell proliferation, ADA2 was considered to belong to a new family of growth factors, named adenosine deaminase-related growth factors (ADGFs) (Zavialov and Engstrom, 2005; Zhang and Takeda, 2007). In contrast to ADA2, there is no much evidence about ADAL functionality so far. However, this ADA member presents conserved catalytically important domains, which may be important for adenosine deamination (Maier et al., 2005; Rosenberg et al., 2007).

Zebrafish (Danio rerio) is a small freshwater teleost widely used in genetic and biochemical studies that combines the relevance of a vertebrate with the scalability of an invertebrate (Goldsmith, 2004; Lieschke and Currie, 2007). It has already been shown that zebrafish genes are highly conserved, since its genome presents similarities with the human genome (Barbazuk et al., 2000). Concerning the purinergic system, studies from our laboratory reported the presence of ecto-nucleotidase activities in zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004) and the differential expression pattern of distinct ADA subfamilies in zebrafish tissues (Rosenberg et al., 2007).

Considering the putative relevance of purinergic signaling in zebrafish brain (Appelbaum et al., 2007; Rico et al., 2008) and that adenosine deamination represents a mechanism able to regulate intracellular and extracellular levels of adenosine/inosine, the goal of this study was to perform a kinetic characterization of ADA activity in soluble and membrane fractions obtained from zebrafish brain.

2. Materials and methods

2.1. Chemicals

Adenosine, 2'-deoxyadenosine, guanosine, 2'-deoxyguanosine, EGTA, EDTA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (EHNA), Coomassie Blue G, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol, sodium nitroprusside, and magnesium chloride were purchased from Merck (Darmstadt, Germany). All other reagents used were from high analytical grade.

2.2. Fish maintenance

Adult wild-type zebrafish (D. rerio), around 3–6 month-old, were obtained from a commercial supplier (Delphis, RS) and acclimated for at least 2 weeks in a 50-L thermostatted aquarium furnished with continuously aerated unchlorinated water. The temperature was kept between 26±2 °C for at least 2 weeks in a 50-L thermostatted aquarium equipped with aerated unchlorinated water. The temperature was kept between 26±2 °C under a 12-h light-dark controlled photoperiod and the animals were fed with commercial fish pellets twice a day. The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.3. Preparation of soluble and membrane fractions

In order to obtain both cellular fractions, zebrafish were euthanized and whole brains were initially homogenized in 20 volumes (v/w) of chilled phosphate buffered saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4 in a glass-Teflon homogenizer. The preparation of brain membranes was according to the method described previously (Barnes et al., 1993). In brief, the homogenate was centrifuged at 800 g for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40000 g. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS and centrifuged for 20 min at 40000 g. This freeze-thaw–wash procedure was used to ensure the lysis of the membrane vesicles. The final pellet was resuspended and used for biochemical assays. Both fractions were maintained at 2–4 °C throughout preparation and they were immediately used for enzyme assays.

2.4. Adenosine deaminase assay

Adenosine deaminase activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported (Weisman et al., 1988). After the preparation of soluble and membrane fractions, the optimum conditions for adenosine hydrolysis were determined. The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for the experiments with soluble and membrane fractions, respectively, in a final volume of 200 μL. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of substrate (adenosine or other, as indicated) to a final concentration of 1.5 mM (with exception for substrate curves). After incubated for 75 min (soluble fraction) and 120 min (membranes) with exception for substrate curves, the reaction was stopped by adding the samples on a 500 μL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/mL). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately mixed to 500 μL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37 °C for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. The ADA activity was expressed as nmol of NH3 min–1 mg–1 of protein.

2.5. Characterization of ADA activity

To verify the influence of temperature, the enzyme assays were carried out under standard assay conditions at various temperatures in the range 25–60 °C. The optimum pH and the effect of divalent cations (Ca2+, Mg2+, and Zn2+) for enzyme activity were assayed as previously described (Mohamed, 2006). The pH dependence was evaluated using 50 mM buffers sodium acetate (pH 3.5–6.0), sodium phosphate (pH 6.5–7.4), and sodium carbonate bicarbonate buffer (pH 8.0–9.0). The effect of divalent cations on ADA activity was tested after preincubation with metals for 10 min. The apparent and Vmax values for adenosine deamination in soluble and membrane fractions were determined from Eadie–Hofstee plots by using substrate concentrations from 0.10 to 3.0 mM.

2.6. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.7. Statistical analysis

All assays were carried out in triplicate and means±SEM of at least four independent experiments were presented. Data were analyzed by student's t-test or one-way analysis of variance (ANOVA), followed by Tukey test as post-hoc. P values ≤0.05 were considered as significant.

3. Results

3.1. Adenosine deamination as a function of time and protein concentration

The adenosine deamination in soluble and membrane fractions of zebrafish brain was evaluated as a function of time and protein concentration (1.5 mM adenosine as initial substrate) in order to determine the correct assay conditions. The soluble and membrane fractions were incubated as described in Materials and methods. The
deamination promoted by ADA activity was linear until the evaluated times: 105 min (soluble) and 180 min (membranes). Furthermore, as the ADA activity during 75 min of incubation (soluble fraction) and 120 min (membranes) was linear in the range of 5–20 μg, we chose to use 5–10 μg of protein from both fractions in the further enzyme assays (data not shown). When both preparations were incubated with their respective times and protein contents without the substrate adenosine, there was no significant production of NH₃. Therefore, the involvement of other NH₃ sources was negligible in the assay condition tested.

3.2. Influence of temperature and pH dependence

To evaluate the influence of temperature on ADA activity, the enzyme assays were carried out in a range of 25–60 °C after a 10 min of preincubation period. The ADA activity increased up to 45 °C (data not shown); thus, the temperature of 37 °C was chosen for further assays in order to lessen the risk of protein denaturation in both cellular fractions. This same incubation temperature was previously used in our laboratory for characterization of ATP, ADP, and AMP hydrolysis in zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004).

The effect of different pH was examined in a range between 5.0–8.0 and 3.5–9.0 for soluble and membrane fractions, respectively. The buffers used were 50 mM sodium acetate (applied in a pH range from 3.5–6.0), sodium phosphate (used in a pH range from 6.5–7.4), and sodium carbonate bicarbonate buffer (assayed from pH 8.0–9.0). The results have shown that the optimum pH for soluble ADA was in a range between 6.0 and 7.0 (Fig. 1A) whereas the optimum pH for membrane-bound ADA was 5.0 (Fig. 1B). Therefore, the pH 7.0 and 5.0 were chosen for the subsequent experiments with soluble and membrane-bound ADA, respectively.

3.3. Effect of divalent cations on ADA activity

In order to investigate a possible effect of divalent cations on ADA activity, 5 mM Ca²⁺, Mg²⁺, and Zn²⁺ were used (n = 5). Zn²⁺ was the only cation able to decrease (by 31%) the ADA activity, specifically from brain membranes, which was prevented by the addition of 5 mM EDTA (Fig. 2). The soluble ADA activity was not altered in the presence of these metals (data not shown).

3.4. Kinetic parameters of zebrafish brain ADA

The adenosine deamination was determined at adenosine concentrations ranging from 0.15 to 3.0 mM in soluble (Fig. 3A) and 0.10 to 3.0 mM in membrane (Fig. 3B) fractions. The apparent Michaelis–Menten constants (Kₘ app) and maximum velocities (Vₘₐₓ app) were estimated from Eadie–Hofstee plots (Fig. 3). The apparent Kₘ were 0.22 ± 0.03 mM (mean ± SEM, n = 6) for soluble and 0.19 ± 0.04 mM (mean ± SEM, n = 6) for brain membrane preparations. The calculated Vₘₐₓ in soluble and membrane fractions were 12.30 ± 0.73 (mean ± SEM, n = 6) and 17.50 ± 0.51
for the regulation of adenosine/inosine levels in distinct cellular presence of these enzymes may be contributing in a different manner has already been reported, demonstrating that ADAL isoform (ADA2-1/T), and ADA1, ADA2, and ADAL, grouped in the adenyl-deaminase family Previous study showed that there are different ADA subfamilies, such as brain (Veauvy et al., 2005). A comparative evaluation of the teleosts, which may cause several deleterious effects in distinct tissues, which also includes reduction in the rate of proteolysis, amino acid catabolism, and a higher conversion to urea in teleosts (Randall and Tsui, 2002). Furthermore, it has been demonstrated that glutamine synthase activity may be regulated according to glutamate and NH3 levels and metabolic activity of fish (Walsh and Milligan, 1995; Wicks and Randall, 2002). Although the tolerance to NH3 toxicity in aquatic animals is considered to be varied, it has been shown that Batrachoididae family are less sensitive to high NH3 levels, possible due to its ability to convert NH3 to less toxic urea or other metabolites, such as glutamine or glutamate (Wang and Walsh, 2000). Considering that distinct ADA members are present in zebrafish, it is plausible that these proteins might be differently contributing for adenosine deamination, and hence, regulating the formation of NH3 in the brain of this species.

In order to verify whether adenosine deamination in zebrafish brain may be altered in the presence of a classical inhibitor of ADA1, both cellular fractions were incubated in the presence and in the absence of 0.1 mM EHNA (Table 2). Although the ADA activities in both soluble and membrane fractions were inhibited by this compound, the inhibition was more pronounced over the soluble (82.3%) than over the membrane-bound (26.5%) ADA activity.

### 3.5. Different nucleoside specificity

The relative substrate specificity of zebrafish brain ADA was determined (Table 1). Adenosine and 2′-deoxyadenosine were the most preferred substrates, compared to guanosine and 2′-deoxyguanosine. The soluble fraction demonstrated a preference for 2′-deoxyadenosine over adenosine, whereas the membrane fraction demonstrated a preference for adenosine. The enzyme activity was higher in the membrane preparation for adenosine (20.9±1.3 nmol NH3 min⁻¹ mg⁻¹ of protein, mean±SEM, n=5) and for 2′-deoxyadenosine (23.6±1.5 nmol NH3 min⁻¹ mg⁻¹ of protein, mean±SEM, n=5) in the soluble fraction.

### 3.6. Inhibition of ADA activity by EHNA

Several studies have demonstrated that EHNA is a potent inhibitor of ADA1 activity (Iwaki-Egawa and Watanabe, 2002; Iwaki-Egawa et al., 2004; Sharoyan et al., 2006). Thus, we measured the adenosine deamination in zebrafish brain in the presence and in the absence of 0.1 mM EHNA (Table 2). Although the ADA activities in both soluble and membrane fractions were inhibited by this compound, the inhibition was more pronounced over the soluble (82.3%) than over the membrane-bound (26.5%) ADA activity.

### 4. Discussion

In the present study, we evaluated the biochemical properties of ADA activity in soluble and membrane fractions of zebrafish brain. Previous study showed that there are different ADA subfamilies, such as ADA1, ADA2, and ADAL, grouped in the adenylyl-deaminase family (Maier et al., 2005). The transcriprional profile of these ADA members has already been reported, demonstrating that ADA1, two paralogues of ADA2 (ADA2-1 and ADA2-2), a truncated ADA2-1 alternative splice isoform (ADA2-1/T), and ADAL are expressed in zebrafish brain (Rosenberg et al., 2007). Therefore, it is possible to suggest that the presence of these enzymes may be contributing in a different manner for the regulation of adenosine/inosine levels in distinct cellular fractions.

The adenosine deamination represents one of the NH3 sources in teleosts, which may cause several deleterious effects in distinct tissues, such as brain (Veauvy et al., 2005). A comparative evaluation of the activity of ammonogenic enzymes, such as AMP deaminase, ADA and glutamate dehydrogenase (GDH) indicated that levels of NH3 in the activity of ammonogenic enzymes, such as AMP deaminase, ADA and glutamate (Wang and Walsh, 2000). Considering that distinct ADA members are present in zebrafish, it is plausible that these proteins might be differently contributing for adenosine deamination, and hence, regulating the formation of NH3 in the brain of this species.

### Table 2

**Inhibition of zebrafish brain ADA by EHNA**

<table>
<thead>
<tr>
<th>Group</th>
<th>ADA activity (mean±SEM) #</th>
<th>Soluble</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.3±1.1</td>
<td>23.1±0.8</td>
<td>16.9±1.0</td>
</tr>
<tr>
<td>EHNA (0.1 mM)</td>
<td>2.0±0.2 *</td>
<td>2.0±0.2</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

* Significantly different from control group (Student’s t-test, p<0.05, n=5).

* The ADA activity was expressed as nmol NH3 min⁻¹ mg⁻¹ of protein.

### Table 1

**Relative substrate specificity of zebrafish brain ADA**

<table>
<thead>
<tr>
<th>Substrate (1.5 mM)</th>
<th>ADA activity (mean±SEM) #</th>
<th>Soluble</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>11.2±0.05 **</td>
<td>20.9±1.3</td>
<td></td>
</tr>
<tr>
<td>2′-Deoxyadenosine</td>
<td>23.6±1.5 **</td>
<td>5.4±0.8</td>
<td></td>
</tr>
<tr>
<td>Guanosine</td>
<td>1.8±0.2 **</td>
<td>1.7±0.2</td>
<td></td>
</tr>
<tr>
<td>2′-Deoxyguanosine</td>
<td>2.0±0.2 **</td>
<td>1.8±0.3</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from adenosine (ANOVA, followed by Tukey test as post-hoc, p<0.05, n=5).

** Significant difference from 2′-deoxyadenosine (ANOVA, followed by Tukey test as post-hoc, p<0.05, n=5).

*ADA activity was expressed as nmol NH3 min⁻¹ mg⁻¹ of protein.
bell-shaped curve, with a decreased about 50% of the activity below pH 5.0 on acidic side and above pH 9.0 on the basic side (Singh and Sharma, 2000). Although the purified human and chicken liver ADA1 have shown the optimal activity in the range of pH 7.4 (Iwaki-Egawa and Watanabe, 2002), it was verified that the ecto-ADA from porcine brain had a very broad optimum pH, in the range 6.0–9.0 (Roma-nowska et al., 2007). Despite to the highest ADA1 activity at pH 7.4, the human ADA2 reaches maximal activity at more acidic pH (Zavialov and Engstrom, 2005). Although there is no evidence about the optimal pH and functionality of ADAL so far, it was demonstrated that this member presents important amino acid residues for adenosine deamination (Maier et al., 2005; Rosemberg et al., 2007). Considering that it is not possible to rule out the contribution of different ADA members for adenosine cleavage in membrane fractions, it is plausible to hypothesize that the higher ADA activity at pH 5.0 could be a result of a combination of different enzyme activities. Moreover, the preference of ADA for acidic pH in zebrafish brain membranes could be physiologically important during stress, hypoxia or brain injury, when adenine levels are significantly elevated and the pH decreases in the extracellular fluid (Sitkovsky et al., 2004; Zavialov and Engstrom, 2005; Eltzschig et al., 2006).

The adenosine deamination in zebrafish brain was also evaluated in the presence of divalent cations, such as Ca2+, Mg2+, and Zn2+. The results have shown that only 5 mM Zn2+ exposure was able to decrease the ADA activity in the membrane fraction. The effect of metals on ADA activity still remains controversial. Previous data showed that zinc may play a critical role for adenosine deamination being located deep within the substrate binding cleft in a tetrahedral geometry (Cooper et al., 1992; Bhaumik et al., 1993). Nevertheless, it was also demonstrated that zinc and other divalent cations are able to interact with other amino acid residues and induce an inhibition of the enzyme activity (Cooper et al., 1992). The characterization of ADA2 from camel tick showed that 5 mM Mg2+ and Zn2+ caused a significant decrease of its activity (Mohamed, 2006). Moreover, it was reported that millimolar concentrations of Zn2+ may also inhibit ADA1 activity, which may be coupled to membrane proteins acting as an ecto-ADA (Cooper et al., 1992; Franco et al., 1997). Our results demonstrated that 5 mM Zn2+ significantly decreased the adenosine deamination in brain membranes, reinforcing the idea that this metal may act as an inhibitor of zebrafish ADA1. The presence of EDTA in the assay solution did not promote significant changes on ADA activity during the assay, indicating that this molecule does not alter the nucleoside binding site or affect protein structure. The presence of 5 mM EDTA plus Zn2+ prevented the inhibitory effect of zinc, which lead us to suggest that it prevented an inhibitory effect of Zn2+ associated at peripheral binding sites since it had been shown that a probable bond metal cofactor is inaccessible to chelators in a high affinity site of ADA protein (Cooper et al., 1992).

The preference to adenine over guanine nucleosides was demonstrated for both soluble and membrane fractions. The soluble ADA displayed a strong preference for 2′-deoxyadenosine as substrate whereas the membrane-bound ADA preferred adenosine over 2′-deoxyadenosine. On the other hand, the guanine nucleosides, guanosine, and 2′-deoxyguanosine were deaminated at considerable lower rates than adenine nucleosides in both cellular fractions. Considering that 1) distinct ADA-related genes have already been identified in zebrafish genome (Maier et al., 2005; Rosemberg et al., 2007); 2) the kinetics of zebrafish ADAL and ADA2-2 still remains unclear; 3) the functionality of a truncated isoform of ADA2-1 is unknown (Rosemberg et al., 2007), it is possible to suggest that the preference for 2′-deoxyadenosine in the soluble fraction may be resulted by deamination activity of different ADA members. Furthermore, it was already demonstrated that the preference for both adenine nucleosides may be varied and that adenosine and 2′-deoxyadenosine are the classical substrates for adenosine deaminase (Iwaki-Egawa and Watanabe, 2002; Iwaki-Egawa et al., 2004).

This study describes the enzymatic and kinetic properties of adenosine deamination in both soluble and membrane preparations from zebrafish brain, which displayed a strong preference for adenine over guanine nucleosides. The presence of ADA activity in the brain membranes suggests the existence of an ecto-ADA in zebrafish. Considering that previous study demonstrated the colocalization of ADA, CD26, and A1 receptors in goldfish brain (Beraudi et al., 2003), it is interesting to hypothesize that they may also be colocalized in zebrafish brain. In conclusion, the adenosine deamination in the CNS of zebrafish promoted by different ADA members may be a key component for controlling the adenosine/inosine levels in both intracellular and extracellular milieu. These data may be important to clarify the mechanisms of adenosine cleavage in zebrafish, which could lead to better understanding about the role of the purinergic system in this species.

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References


