Characterization of a novel *Drosophila melanogaster* acylphosphatase

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Abstract **Analysis of the *Drosophila melanogaster* EST database led to the characterization of a novel acylphosphatase (AcPDro2). This is coded by the CG18505 (Acyp2) gene and is clearly distinct from a previously described AcPDro coded by the CG16870 (Acyp) gene from *D. melanogaster*. The two proteins show a 60% homology with both vertebrate isoenzymes. All the residues involved in the catalytic mechanism are conserved. AcPDro2 is a stable enzyme with a correct globular folded structure. Its activity on benzoylphosphate shows higher K_{cat} but lower K_m with respect to AcPDro. It is possible that AcPDro and AcPDro2 genes are not the direct ancestor of MT and CT vertebrate isoenzymes.**

Key words: Acylphosphatase; Activity; Protein stability; Ancestor; *Drosophila*

1. Introduction

Acylphosphatase (E.C. 3.6.1.7) is a small cytosolic enzyme which catalyzes the hydrolysis of carboxyl phosphate bond of synthetic and physiological molecules [1]. The role and the function of this enzyme in vivo is not yet completely clear though a number of in vitro evidences show that acylphosphatase hydrolytic activity has an effect on membrane pumps that present phosphointermediates in their catalytic cycle, suggesting a possible role in controlling the ion transport across biological membranes [2–4]. Other evidences show an increasing level of expression during differentiation of several human cell lines [5–7]. In addition, it has been demonstrated that overexpression of acylphosphatase in HeLa cells induces apoptosis [8].

Acylphosphatase is widely distributed in many tissues of vertebrate species and it is found as two isoforms, named muscle-type (MT) and common-type (CT) sharing a 56% of sequence identity. The characterization of isoforms from phylogenetically representative organisms shows a stable conservation of amino acid sequence from remote vertebrates to mammalians [1].

The three-dimensional structure of both isoenzymes, determined by NMR and X-ray crystallography, shows a similar conformation, a typical α/β globular fold found in other phosphate binding proteins [9,10]. Recently, it was demonstrated that acylphosphatases are able to form protein aggregates morphologically similar to those involved in degenerative pathologies such as Alzheimer's disease and Parkinson's disease [11]. At present acylphosphatases are considered a good model system for protein aggregation studies [12,13].

Site directed mutagenesis demonstrated that the Arg-23 conserved residue is responsible for the phosphate binding and that the Asn-41 residue is responsible for catalytic activity. The proposed catalytic mechanism considers the orientation of a catalytic water molecule by the Asn-41 residue towards the substrate and the subsequent disruption of phosphate bond from acyl group and phosphate group [1].

Although acylphosphatases have been isolated and characterized only from vertebrate species, the detection of the acylphosphatase activity has been reported in lower organisms such as bacteria and insects. Also characterized was a *Drosophila melanogaster* acylphosphatase (named AcPDro), as the product of a single exon in the Acyp gene located in 2L chromosome. This enzyme shows a 40% identity and a 66% homology with both human isoenzymes, though presenting an additional tail of 22 amino acids at the C-terminus. Since it was the most phylogenetically distant acylphosphatase that was characterized, it was considered as the possible common ancestor for MT and CT vertebrate isoenzymes [14].

In this paper we report the characterization of the protein coded by the CG18505 (Acyp2) gene. This work demonstrates that the product of Acyp2 gene is a fully active enzyme. This is a novel acylphosphatase (AcPDro2), with kinetic properties clearly distinct from those of the previously described AcPDro [14]. The presence of more than one protein with acylphosphatase activity in *D. melanogaster* demonstrates that also in this species more than one form of this enzyme is present.

2. Materials and methods

2.1. Materials

Benzoylphosphate was synthesized as previously described [15] and freshly dissolved before enzyme activity measurements. The vector containing the CG18505 (Acyp2) cDNA was from ResGen™ (Invitrogen Corporation). Restriction enzymes, pGEX 4T-1 expression vector, IPTG and glutathione–Sepharose affinity matrix were from Amersham Biosciences. Oligonucleotides and bacterial hosts (DH5α) for propagation of plasmids and expression of the recombinant protein were from Invitrogen. The plasmid extraction kit was from Macherey-Nagel. PCR purification and other DNA purification steps were made with Quiagen kits. Bovine thrombin was from Sigma. All other reagent were of analytical grade or the best available commercially.

2.2. PCR amplification and cloning

The search for putative acylphosphatase was performed using the human acylphosphatase amino acid sequences as a query in the Gen-

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Bank database using the BLASTp program [16]. The GadFly database was then mainly used, together with InterPro and EBI databases.

The amplification of the bank CG18505 (Acyp2) gene corresponding DNA coding region (obtained from ResGen™ *Drosophila* cDNA clone) was performed using the forward primer DRODir (5′-GTTCC GCGTG GATCC GCGGG ATCTG GAGTTG), containing *Bam*HI restriction site and the reverse primer DRORev (5′-GCGGC GCTCG AGTTA ATGTT TTATG TCAAA GGAAGT), containing *Xho*I restriction site. After a 30-cycle amplification (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) the product, with the expected size of about 300 bp, was purified with MinElute kit. Both pGEX 4T-1 vector and PCR product were digested with *Bam*HI and *Xho*I restriction enzymes. The vector was then treated with alkaline phosphatase to avoid plasmid self-ligation. The PCR fragment was ligated into *Bam*HI and *Xho*I sites of the digested pGEX 4T-1 vector, downstream and in frame with the glutathione S-transferase coding region present in the vector and amplified in DH5α *Escherichia coli* strain.

GST-AcPDro2 expressing clones were checked by inducing liquid cultures with 0.1 mM IPTG. A centrifugation step and a lysis of pellets directly in denaturing Laemmli sample buffer was used to obtain samples of total proteins from each positive clone. These was analyzed in a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The plasmid of the positive clone expressing the greatest amount of fusion protein was extracted and automatically sequenced.

2.3. Expression and purification of AcPDro2

The GST-AcPDro2 protein was expressed in DH5α-*E. coli* by 0.1 mM IPTG induction, purified through a glutathione–Sepharose affinity chromatography and finally subjected to thrombin cleavage. The purity of cleaved protein was checked on 15% SDS–PAGE and its exact mass was checked in electrospray mass spectrometer. Protein concentration was determined by measuring its 280 nm absorbance.

2.4. Enzymatic activity assays

Acylphosphatase activity was determined by a continuous optical test at 283 nm using benzoylphosphate as a substrate. pH optimum was checked using a 5 mM concentration of substrate in different buffers (acetate 0.1 M pH ranging from 3 to 6, cacodylate 0.1 M pH ranging from 6 to 7 and Tris 50 mM pH ranging 7 to 8). Enzymatic characterization was performed at 25°C in 0.1 M acetate buffer pH 5.3 and 0.05 mg/ml bovine serum albumin as stabilizing factor, since activity loss was observed in diluted enzyme. The plot of enzymatic activity versus substrate concentration was analyzed using the Michaelis–Menten equation, with the Kaleidagraph software.

2.5. Urea-induced denaturation

All equilibrium fluorescence measures were carried out on a Perkin–Elmer spectrofluorometer equipped with a thermostated water-circulating bath. Excitation wavelength was set to 280 nm, emission wavelength was adjusted to 350 nm. Urea stock solutions were freshly prepared. Denaturation data were measured at 28°C after 1 h incubation of the protein at a concentration of 0.02 mg/ml in 10 mM acetate buffer pH 5.3, with the concentration of urea ranging from 0 to 8.5 M. Fluorescence signals were plotted as a function of the urea concentration and the data were directly fitted to the equation reported by Santoro and Bolen [17]. All data and plots were treated with Kaleidagraph software.

3. Results

Search in gene bank GadFly *D. melanogaster* reports some sequences coding for putative acylphosphatases. The *Drosophila* CG18505 gene (Acyp2) is located in the 3R chromosome, mapping in the 89A8 band. Because of the similarity between the putative coded acylphosphatase and previously characterized human isoenzymes, we decided to clone the corresponding cDNA and attempt the production of such a protein. Transcription of this gene is granted by the presence of the cDNA in *Drosophila* gene bank GadFly.

We named the protein AcPDro2. It shares a 37.7% and a 42.8% identity when compared with human MT and CT isoenzymes respectively (Fig. 1), a very similar degree of identity with respect to that of AcPDro, as already described [14].

We obtained the *Drosophila* cDNA clone harboring AcPDro2 from ResGen™ (Invitrogen Corporation). With the use of PCR technique, described in Section 2, we obtained the coding region of this acylphosphatase. This coding sequence was inserted in the pGEX 4T-1 vector, downstream the GST (glutathione S-transferase), in order to obtain AcPDro2 expression in *E. coli* as fusion protein.

The expression, the purification of the GST-AcPDro2 fusion protein and the subsequent cleavage with thrombin gave a typical yield of 4 mg of pure AcPDro2 protein per liter of culture. The purity of AcPDro2 was checked and the exact mass was confirmed by electrospray mass spectrometry, resulting in 11 778 Da in agreement with the theoretical determination based on amino acid sequence.

Fig. 2a shows the main enzymatic characteristics of the purified enzyme using benzoylphosphate as substrate and inorganic phosphate as competitive inhibitor. AcPDro2 hydrolyzes benzoylphosphate at a pH optimum comparable to that of human isoenzymes, but the K_m is higher with respect to human isoenzymes and AcPDro. The K_{cat} value, on the other hand, is lower than that of the human isoforms but much higher with respect to AcPDro. All these data are summarized in Table 1. The K_{cat}/K_m ratio indicates that AcPDro2 is a more efficient enzyme with respect to AcPDro. These values were obtained using benzoylphosphate, the synthetic substrate used to assay the acylphosphatase activity.

The typical two-state transition observed on urea-induced denaturation at equilibrium (Fig. 2b) revealed that AcPDro2 possesses a well-defined globular structure. The analysis of the denaturation curve indicates that AcPDro2 is a less stable enzyme ($C_m = 2.52$ M) than human acylphosphatases [11], though maintaining a conformational stability higher than that of AcPDro [14], as shown in Table 2.

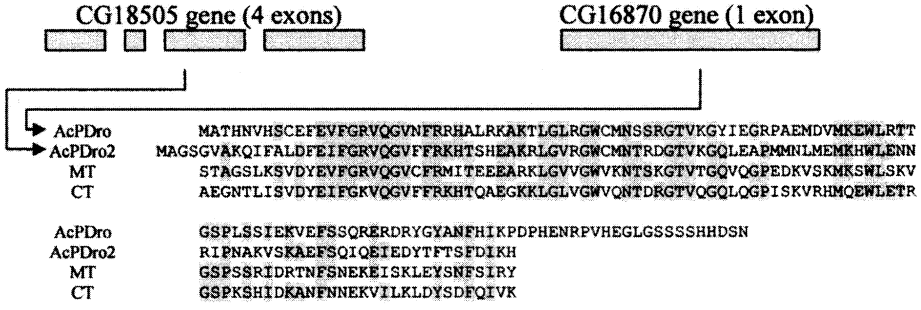

Fig. 1. Amino acid sequence alignment of both CT and MT human isoenzymes and *Drosophila* acylphosphatases. The *Drosophila* enzymes derive from different genes, with different intron–exon organization and located into different chromosomes.

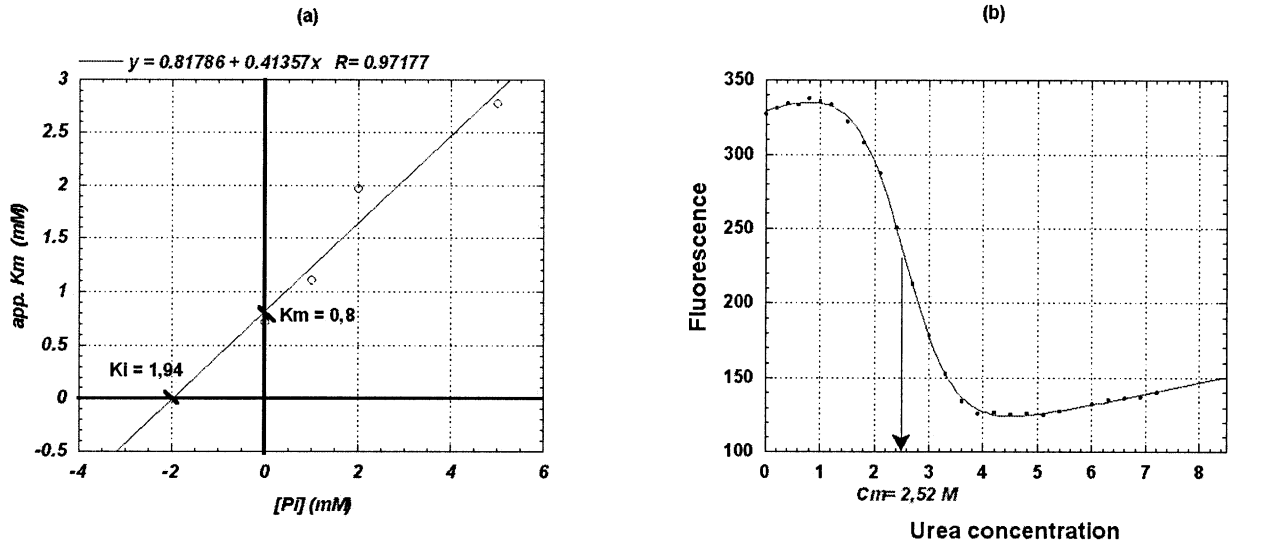

Fig. 2. Kinetic assays conducted over AcPDro2. a: Secondary plot of K_m and K_i deriving from non-linear fitting of data with Michaelis–Menten equation. b: Urea-induced denaturation assay with the Santoro and Bolen non-linear fitting of data. The arrow indicates the C_m value of AcPDro2.

4. Discussion

In 1998 the AcPDro enzyme of *D. melanogaster* was characterized [14]. At that time it was supposed that its gene might represent a common ancestor for vertebrate isoforms, since no other isoform was known in *Drosophila*. In the last years complete sequencing of the *Drosophila* genome has been performed [18]. This fact led us to find some new gene coding for putative acylphosphatases. We selected to clone the CG18505 (Acyp2) gene because it encodes a protein with a degree of homology with human acylphosphatases and AcPDro higher than that exhibited by other putative acylphosphatase proteins from *D. melanogaster*, taking also in account the protein length.

The amino acid sequences alignment of the two enzymes of *D. melanogaster* with the human isoforms (taken here as examples of vertebrate enzymes) shows that the key residues of acylphosphatases (the substrate positioning loop 15–21 and the catalytic residues Arg-23 and Asn-41, in human isoenzymes) are conserved in both fruit fly forms. Important differences between AcPDro2 and the previously identified AcPDro can be observed. First, their genes are located on different chromosomes and their genes are structured as a single and a four exon gene, respectively (Fig. 1). The amino acid similarity suggests that the two genes may have origin in a common ancestor, possibly through gene duplication and further mutations.

AcPDro2 is more similar to the vertebrate isoforms as it is composed of 102 amino acids. This contrasts with the length of 122 amino acids of AcPDro (Fig. 1). Removal of the 22 amino acid tail at the C-terminus of AcPDro was shown to leave the enzymatic characteristics substantially unchanged, the only modification being a lower substrate binding affinity [14].

The results presented here show that the CG18505 (Acyp2) gene encodes a protein with a precise and stable folded state, which presents a fully active acylphosphatase activity. This novel protein is a more efficient enzyme with respect to AcPDro. Although it displays a lower capability to bind the substrate and its inhibitor, the higher catalytic efficiency to hydrolyze the substrate contributes to make the K_{cat}/K_m value considerably higher than that of AcPDro. However, AcPDro2 catalytic efficiency is lower than that of the vertebrate enzymes. Unlike AcPDro, AcPDro2 is expressed in a larval phase of the *Drosophila* life, as demonstrated by the presence of its cDNA in larval GadFly database [19]. It could therefore play a role in the development of this organism.

It should be underlined that the fruit fly acylphosphatase forms so far characterized share a similar sequence identity score with both CT and MT vertebrate enzymes. This fact suggests that the divergence between CT and MT genes on one hand and AcPDro and AcPDro2 genes on the other hand may derive from distinct evolutionary pathways. The presence of at least two different acylphosphatase enzymes in *Drosophila* has lead us to doubt about the previous hypothesis on the identification of AcPDro as a common ancestor of the two vertebrate acylphosphatases isoenzymes.

More acylphosphatases in *Drosophila*, as well as in vertebrates, seem to underline the importance of such an enzyme activity through evolution.

Table 1
Main enzymatic characteristics of human and *Drosophila* acylphosphatases (all data contain an experimental error of approximately 10%)

Enzyme	K_{cat} (s^{-1})	pH opt.	K_m (mM)	K_i (mM)	K_{cat}/K_m (s^{-1} mM^{-1})
Human CT-AcP	1420	5.0–6.0	0.15	0.58	9470
Human MT-AcP	1230	4.8–5.8	0.36	0.75	3420
AcpDro	35	5.3–6.3	0.18	0.38	194
AcpDro2	735	4.8–5.8	0.80	1.94	920

Table 2
Main thermodynamic parameters of human and *Drosophila* acylphosphatases (all data contain an experimental error of approximately 10%)

Enzyme	$\Delta G(H_2O)$ (kJ mol^{-1})	m (kJ mol^{-1}M^{-1})	C_m (M)
Human CT-AcP	22.0	6.0	4.02
Human MT-AcP	21.3	5.3	4.54
AcpDro	10.3	6.5	1.58
AcpDro2	13.9	5.5	2.52

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