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Drosophila melanogaster acylphosphatase: a common ancestor for acylphosphatase isoenzymes of vertebrate species

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Abstract **An open reading frame encoding a putative acylphosphatase was found in *Drosophila melanogaster*. The corresponding gene product shows 40% identity and 22 additional amino acid residues at the C-terminus as compared to muscle- and common-type human acylphosphatases. Moreover, all the residues involved in the catalytic mechanism of vertebrate enzymes are conserved in the *D. melanogaster* acylphosphatase. The *D. melanogaster* protein and a deletion mutant, similar in length to vertebrate acylphosphatases, were produced by cloning the corresponding cDNA in *Escherichia coli*. The wild-type enzyme is a protein with a well-established three-dimensional fold and a markedly reduced conformational stability as compared to vertebrate isoenzymes. The specific activity of the enzyme is significantly lower than that found in vertebrate enzymes though the substrate binding capability is basically unaltered. The deletion of 22 residues does not cause a significant change in k_{cat}, while affecting the apparent binding parameters. This work suggests that the genes encoding the vertebrate enzymes originate from an ancestor gene by duplication and subsequent evolution.**

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Key words: Acylphosphatase; Human muscle acylphosphatase; *Drosophila*

1. Introduction

Acylphosphatase (EC 3.6.1.7) is a small cytosolic enzyme, which catalyses the hydrolysis of the carboxyl phosphate bond of molecules both synthetic and of physiological interest [1]. The function in vivo of the enzyme is still not completely understood though a number of in vitro data concerning acylphosphatase hydrolytic activity on the phosphointermediate formed during the catalytic cycle of membrane pumps of several tissues have been collected, suggesting a possible role of the enzyme in the control of ion transport across biological membranes [2–4]. Another line of research demonstrated an increase of acylphosphatase expression levels during differentiation of several cell lines [5,6]; in addition, acylphosphatase overexpression in K562 cells induces a clear increase of differentiated cells after differentiative exposure [6].

Acylphosphatase is widely distributed in many tissues of vertebrate species, where it is found as two isoforms, named muscle-type and common-type isoenzymes [7,8]. The comparison of the primary structure of each isoform from different sources reveals that both isoenzymes are highly conserved. The two human isoforms share 56% sequence identity; this identity increases to 62% and 69% in avian and bony fish species, respectively [1]. The relative abundance of acylphosphatase rises from ancient to more recent vertebrate species suggesting that a progressively more important role of this enzyme has been achieved during phylogenesis [9]. To date, acylphosphatase has only been isolated and characterised from vertebrate species, although the detection of a possible acylphosphatase activity has been reported in lower organisms, such as bacteria, yeast and insects [1].

The three-dimensional structures of the two isoforms, as determined by NMR and X-ray crystallography [10,11], appear very similar to each other showing a typical α/β globular fold found in other phosphate binding proteins [1]. Acylphosphatase structure and site-directed mutagenesis experiments led to the proposal of a substrate-assisted catalytic mechanism [11]. In particular, the conserved residues Arg-23 and Asn-41 have been recognised as the main phosphate binding site and the catalytic residue involved in the orientation and stabilisation of catalytic water molecule, respectively [12,13]. The importance of the invariant residues belonging to the 15-21 and 42-45 loops and the role of the C- and N-terminal regions in enzyme catalysis and conformational stabilisation have also been investigated [14,15].

The cloning and expression of the first acylphosphatase gene from a non-vertebrate source is described in this paper. The kinetic and structural characterisation of the gene product, together with the evolutionary analysis, demonstrates that the *Drosophila melanogaster* acylphosphatase can be considered an intermediate form between the muscle- and common-type isoenzymes, supporting the hypothesis of a duplication and divergent evolution of a common ancestor gene.

2. Materials and methods

2.1. Materials

Benzoylphosphate was synthesised as previously described [16] and freshly dissolved before enzyme activity measurements. Restriction and modification enzymes, oligonucleotides, pGEX-2T plasmid and IPTG were from Pharmacia Biotech. [γ-^{32}P]ATP and [α-^{32}P]ATP (800 Ci/mmol) were from New England Nuclear. Sequenase was from USB. Plasmid propagation and recombinant fusion protein expression were achieved by using DH5α and TB1 bacterial strains, respectively. Glutathione, bovine thrombin and glutathione-agarose affinity gel were from Sigma. D_2O and d_4-acetic acid were from Merck. All other reagents were analytical grade or the best commercially available.

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Abbreviations: MT-, CT-AcP and AcPDro, muscle-type, common-type and *Drosophila melanogaster* acylphosphatase, respectively; Δ22, *Drosophila melanogaster* acylphosphatase deletion mutant; IPTG, isopropylthiogalactoside; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UTR, untranslated region

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2.2. RT-PCR amplification and cloning

The search for DNA sequences encoding for putative acylphosphatase was performed using the human acylphosphatase as query sequence in the GenBank data base with the BLAST program [17]. Total RNA was prepared from *D. melanogaster* by the acidic guanidinium isothiocyanate-phenol-chloroform extraction method [18]. First strand cDNA synthesis was carried out with oligo(dT); DNA amplification was performed by PCR, using two primers designed on the basis of the 5′ and 3′ ends of the ORF (DROS-DIR and DROS-REV, respectively), the first primer containing the restriction site for *Bam*HI to obtain a correct insertion in the pGEX-2T plasmid. After 30 cycles amplification (94°C for 30 s, 65°C for 30 s and 72°C for 30 s), the PCR product with the expected size (about 380 bp) was cloned into pCRII plasmid (Invitrogen) and sequenced by dideoxy chain termination [19] using Sequenase II polymerase (USB) in order to exclude point mutations possibly occurred during PCR. The cDNA insert obtained from pCRII plasmid by digestion with *Xba*I and *Hin*dIII was treated with Klenow and finally digested with *Bam*HI. This fragment was ligated into the *Bam*HI site of the pGEX-2T vector, downstream and in frame with the glutathione *S*-transferase coding region, and amplified in DH5α bacterial strain.

2.3. Northern blot analysis

Total RNA was prepared as described above, separated on 1% denaturing formaldehyde agarose gel and transferred to a nylon filter. The coding region of the *D. melanogaster* acylphosphatase (AcPDro) cDNA, labelled by the random primer method (Stratagene), was used as a probe. Hybridisation was carried out with 1×10^8 cpm/μg of probe at 65°C for 18 h. The size of the resulting band was estimated by comparison with RNA standards (0.28–1.77 kb RNA ladder).

2.4. Site-directed mutagenesis

A deletion mutant lacking 22 amino acid residues at the C-terminus (Δ22) was obtained by inserting a stop codon (TAG) instead of the GAT codon for the Asp-98 using the USE mutagenesis Kit (Pharmacia) according to Deng and Nickoloff [20]. The mutagenised double-stranded DNA was introduced by transformation into *Escherichia coli* DH5α strain and mutation was confirmed by DNA sequencing [19].

2.5. Expression and purification of proteins

The recombinant *D. melanogaster* and human enzymes were expressed in *E. coli* TB1 strain as previously described with minor modifications [21]. All acylphosphatases were produced as fusion proteins with glutathione *S*-transferase and subsequently subjected to thrombin cleavage and further purification as reported by Fiaschi et al. [21]. Protein concentration was determined using the Bradford assay (Bio-Rad) and the purity of proteins was checked on 15% SDS-PAGE.

2.6. Enzymatic activity assay and kinetic characterisation

Acylphosphatase activity was determined by a continuous optical test at 283 nm and 25°C using benzoylphosphate as a substrate dissolved in 0.1 M acetate buffer pH 5.3, as previously reported [22]. For pH optimum determination, acylphosphatase activity assays were performed in 0.1 M acetate or 50 mM 3,3-dimethylglutarate buffer in the pH range 3.7–7.7.

2.7. ^1H NMR experiments

One-dimensional ^{1}H NMR experiments were performed at 600 MHz on a AMX600 Bruker spectrometer, at 25°C as previously described [12]. The samples were prepared by five dilution/concentration cycles in 20 mM phosphate/D_2O buffer, pH 7.0, containing 50 mM NaCl, using Centricon-3 microconcentrators (Amicon) at a final protein concentration of 0.3–0.5 mM. 1,4-Dioxane was used as an internal chemical shift reference at 3.74 ppm.

2.8. Urea-induced denaturation

All equilibrium fluorescence measurements were carried out on a Shimadzu Model RF-5000 spectrofluorophotometer equipped with a thermostatted water-circulating bath, using a 10 mm pathlength quartz cuvette. The excitation and emission wavelengths were 280 nm and 335 nm, respectively. Urea stock solutions were freshly prepared. Urea denaturation curves were obtained at 25°C by recording the fluorescence signal of 1 h preincubated samples containing 0.02 mg/ml protein in 50 mM 3,3-dimethylglutarate buffer, pH 7.0 and urea concentrations ranging from 0 to 8 M. Plots of fluorescence signal as a function of urea concentration were directly fitted to the equation reported by Santoro and Bolen [23]. Reversibility of unfolding was checked by measuring the intrinsic fluorescence of a sample obtained by dilution of urea denatured enzyme immediately after the dilution. Data were analysed by the Kaleidagraph program (Abelbeck, UK).

3. Results

A *D. melanogaster* ORF, whose corresponding protein shows a high sequence homology with vertebrate acylphosphatase, was found in the GenBank data base, accession number AC001660, mapping in the alcohol dehydrogenase region of chromosome 2. Alignment of the putative *D. melanogaster* enzyme (AcPDro) and the vertebrate acylphosphatase sequences is shown in Fig. 1. AcPDro is composed of 119 amino acid residues, displaying 21 additional residues compared to the vertebrate isoforms: AcPDro lacks the first residue at the N-terminus and shows a 22 amino acid tail at the C-terminus. AcPDro reveals a notable sequence identity, averaging 40%, to both muscle- and common-type acylphosphatase isoenzymes and this identity reaches about 66% when conservative substitutions are considered. Interestingly, the highest sequence conservation is observed for the regions whose importance in vertebrate AcP has been previously demonstrated [12–14]. In particular, the sequence corresponding to the loop 15-21 (14-20 in AcPDro), involved in substrate positioning, and the two catalytic residues Arg-23 and Asn-41 (Arg-22 and Asn-40 in AcPDro) are conserved. In the light of such intriguing sequence similarities we decided first to verify the transcription of such ORF in the cell and, afterwards, to attempt the production in *E. coli* of both wild-type AcPDro and a 22 amino acid C-terminus deleted mutant (Δ22). RT-PCR performed on total *D. melanogaster* RNA resulted in the amplification of the entire coding sequence as confirmed by sequencing. Northern blot analysis, carried out on total RNA, reveals a single band transcript of approximately 0.8–0.9 kb in length (data not shown). An RT-PCR experiment, performed with DROS-DIR oligonucleotide and an oligo(dT) primer,

Table 1
Main kinetic parameters calculated from activity measurements

Enzyme	k_{cat} (s^{-1})	pH optimum	K_m (mM)	K_i (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
AcPDro	35	5.3–6.3	0.18	0.38	194.4
Δ22	29 (37[a])	6.3–7.3	1.50 (1.54[a])	2.46	19.3 (24.0[a])
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

K_m and k_{cat} were determined using benzoylphosphate as substrate in 0.1 M acetate, pH 5.3. K_i was determined using inorganic phosphate as competitive inhibitor. The pH optimum determination was carried out in 0.1 M acetate and 50 mM 3,3-dimethylglutarate buffers at a pH ranging from 3.7 to 7.7. Results are the mean of four different determinations.

[a]Kinetic parameters determined by acylphosphatase assay performed in 50 mM 3,3-dimethylglutarate, pH 6.5.

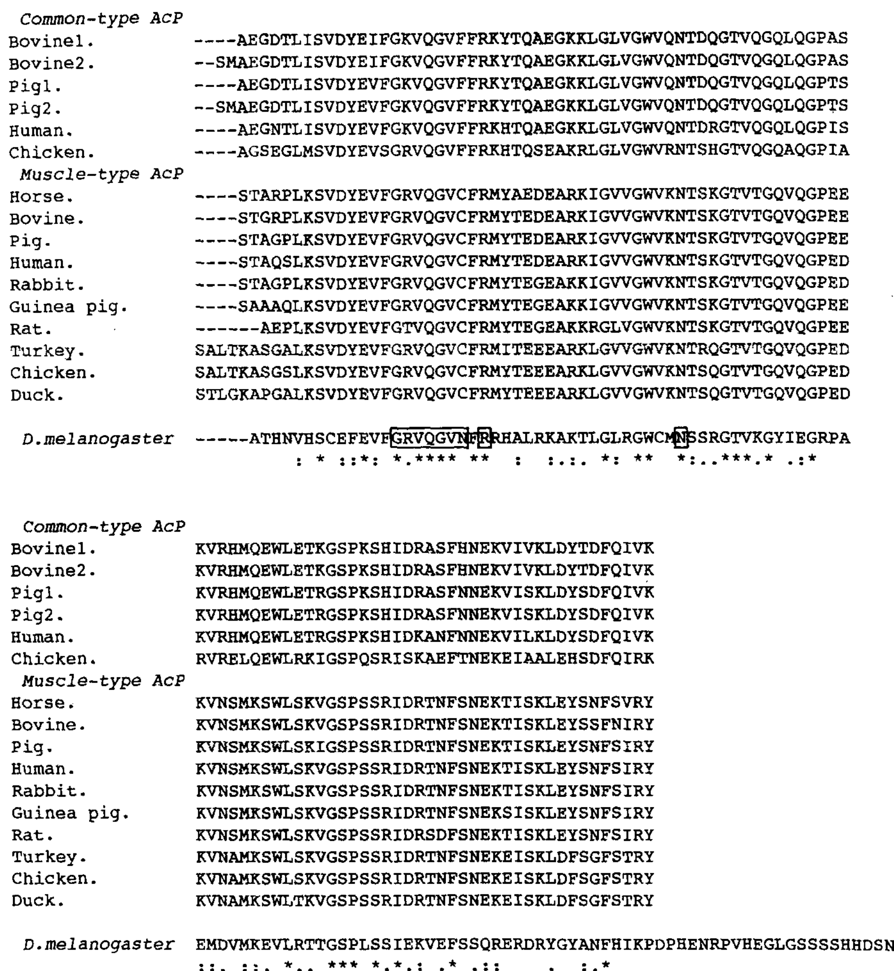

Fig. 1. Sequence alignment of *D. melanogaster* acylphosphatase and acylphosphatase isoenzymes from several vertebrate species. The amino acid sequences of common- and muscle-type acylphosphatases are from Stefani et al. [1]. Data processing was performed on line using the ClustalW program at the WWW Service of the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/) [29]. The residues involved in the formation of the active site are boxed. An asterisk indicates identical residues in all sequences; colons and dots indicate the positions at which conservative and semiconservative substitutions are found, respectively.

resulted in the amplification of a 500 bases long fragment (data not shown): from this it can be deduced that the approximate length of the 3′-UTR is 120–140 bases and, consequently, the 5′-UTR should be about 3–400 bases in length.

The wild-type protein and a deletion mutant lacking the 22 exceeding C-terminal residues with respect to vertebrate isoforms was expressed in *E. coli* giving a typical yield of 4 mg of pure protein per litre of culture. The purity of AcPDro and Δ22 was assessed by 15% SDS-PAGE (data not shown) and the relative molecular mass of the wild-type protein, as determined by electrospray mass spectrometry, resulted to be 13 711 Da, in agreement with theoretical calculations (13 710.24 Da).

Table 1 summarises the main kinetic parameters determined for the two purified enzymes using benzoylphosphate and inorganic phosphate as a substrate and competitive inhibitor, respectively. AcPDro shows hydrolytic activity on benzoylphosphate with the optimum of pH in a range comparable to that of human isoenzymes (Table 1). However, the wild-type enzyme reveals a drastically reduced k_{cat} (lower than 3%) compared to human isoenzymes, whereas the binding parameters, as calculated by non-linear fitting to the Michaelis-

Table 2
Main thermodynamic parameters calculated from urea denaturation experiments

Enzyme	$\Delta G(H_2O)$ (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	C_m (M)
AcPDro	10.3	6.5	1.58
Δ22	10.5	7.0	1.50
Human CT-AcP	22.0	6.0	3.67
Human MT-AcP	21.8	4.8	4.54

Thermodynamic parameters were determined by urea denaturation experiments carried out in 50 mM 3,3-dimethylglutarate buffer, pH 7.0. $\Delta G(H_2O)$ is the free energy change of unfolding, m is the free energy dependence on urea concentration and C_m is the urea concentration at which the enzyme molecules are 50% unfolded. The results are the mean of three different determinations. Experimental errors are ca. 0.10 M for C_m, 0.4 kJ mol^{-1} M^{-1} for m and 5% for $\Delta G(H_2O)$.

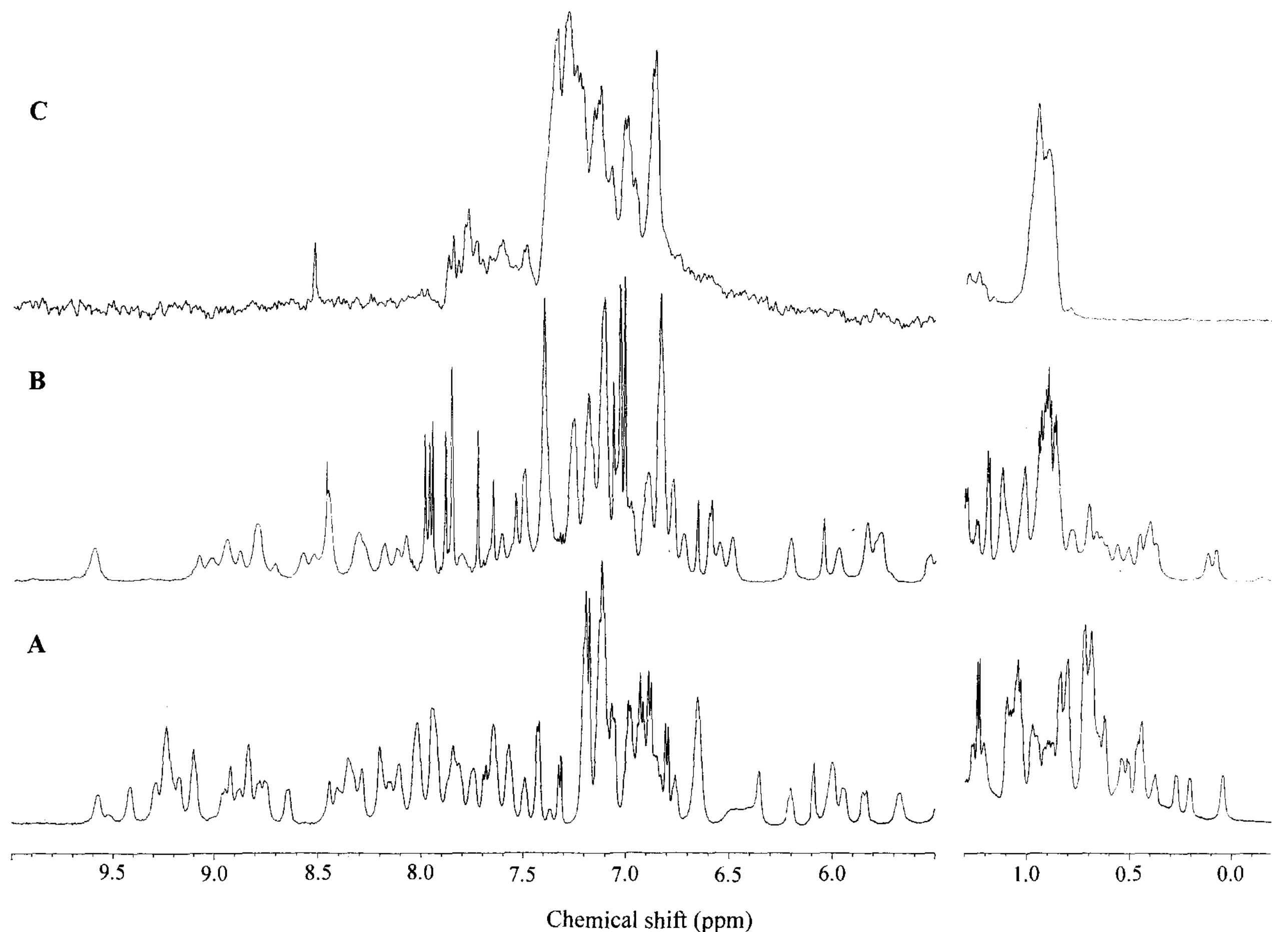

Fig. 2. Aliphatic and aromatic regions of the 600 MHz ^{1}H NMR spectra of acylphosphatases, recorded in 50 mM phosphate/D_2O buffer, pH 7.0. A: Human muscle-type acylphosphatase at 25°C. B: *D. melanogaster* acylphosphatase at 25°C. C: Thermally denatured AcPDro at 70°C.

Menten equation, appear remarkably similar to those of human acylphosphatases (Table 1). The k_{cat}/K_m parameter reflects a strongly reduced catalytic capacity toward the hydrolysis of benzoylphosphate. The kinetic analysis of the Δ22 reveals that the additional C-terminal tail does not play a primary role in the enzyme catalysis, as underlined by the very similar k_{cat} values observed for the two enzymes. The pH optimum for Δ22 activity on benzoylphosphate was found to be 6.3–7.3, a range different from that observed for AcPDro and the vertebrate enzymes. However, the binding affinity of the mutant enzyme is significantly reduced both at pH 5.3 and 6.5 (Table 1). These data account for a possible involvement of the C-terminal tail in substrate binding.

The ^{1}H NMR spectrum (Fig. 2B) of AcPDro shows a remarkable chemical shift dispersion typical of a protein with a well-constrained tertiary fold [24]. Moreover, the amide proton resonances (7.5–9.5 ppm) are still present, as is expected for a protein with a persistent and defined three-dimensional structure [24]. The comparison of the ^{1}H NMR spectra of AcPDro and muscle-type AcP reveals substantial differences between the two proteins; however, a careful inspection of the 5.5–6.5 ppm region indicates a surprising similarity in the number and dispersion of the resonances (Fig. 2A,B). This region, in the ^{1}H NMR spectrum of muscle-type AcP, is characterised by the presence of peaks due to α protons involved in β-sheet structure [25].

Both the wild-type and Δ22 proteins undergo a typical two-state transition, as determined by the urea-induced denaturation curves (data not shown) with a strongly reduced resistance to urea denaturation, as indicated by the low C_m values (Table 2), when compared to either the muscle- or the common-type isoenzymes. $\Delta G(H_2O)$ values, reflecting the conformational stability of both *D. melanogaster* enzyme and Δ22

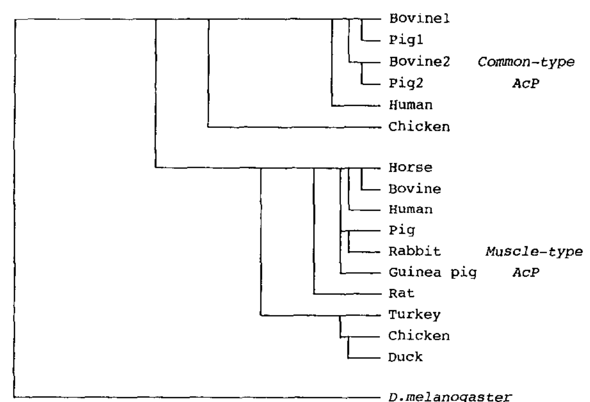

Fig. 3. Phylogenetic tree prediction of acylphosphatase enzymes is based on the multiple sequence alignment reported in Fig. 1 and has been achieved according to Chumakov and Yushmanov (http://www.genebee.msu.su/services/phtree_reduced.html) [30].

mutant, are also markedly reduced as compared to the human isoforms (Table 2). Interestingly, the behaviour of wild-type and mutant *D. melanogaster* enzymes in the urea denaturation experiments are superimposable giving rise to very similar free-energy difference values for the unfolding process and indicating that the presence of the 22 residues C-terminal tail does not significantly affect the overall conformational stability of the enzyme. Dilution of the urea denatured enzymes determined the immediate recovery of over 80% of the fluorescence signal of the native enzyme, indicating that the urea induced unfolding is substantially reversible.

4. Discussion

To date acylphosphatase has been found only in organs and tissues of vertebrate species [1]. The cloning and expression of a *D. melanogaster* ORF coding for a putative acylphosphatase which shows a high degree of identity with respect to vertebrate isoforms and the conservation of the catalytically relevant residues enable us to shed light on the molecular evolution of acylphosphatase.

As far as the entire coding region for AcP is concerned, it can be noticed that no intervening sequence is present in the *D. melanogaster* gene, the cDNA sequence obtained by RT-PCR being identical to that present in the genomic clone. Moreover, it can be observed that nearly half of the single mRNA transcript coding for AcPDro is unusually covered by the 5′-UTR. A very similar situation has been found for the human MT-AcP mRNA where it was also evidenced that the 5′-UTR plays a role in the stability of the mRNA molecule [26].

The expression of the AcPDro ORF in *E. coli* allowed the corresponding protein to be purified: the kinetic parameters determined for this product reveal a significant similarity to those calculated for human acylphosphatases, with the exception of k_{cat}, which, in contrast, appears drastically reduced. However, AcPDro maintains a significant acylphosphatase activity. The ^{1}H NMR experiments, performed on AcPDro, in order to exclude a partial denaturation of the enzyme possibly responsible of the reduced acylphosphatase activity, reveal a notable chemical shift dispersion and a significant protection of labile protons against exchange with the deuterated solvent, indicating the presence of a well established fold, typical of a native protein.

Since the vertebrate enzymes are generally constituted of a 98 amino acids long polypeptide chain, it could not be excluded that the additional 22 C-terminal amino acid residues in the *D. melanogaster* enzyme are responsible for the drop of the catalytic efficiency. However, the Δ22 mutant shows a k_{cat} value very similar to that of AcPDro, indicating that the C-terminal region is not directly involved in catalysis. This result is in agreement with previous evidences on human muscle-type acylphosphatase mutants, which indicated that the presence of two or four additional residues to the C-terminus induces only a partial decrease of the specific activity [15]. The lower catalytic activity observed in AcPDro as compared to human isoenzymes might be, therefore, attributed to local differences in their three dimensional structures and, particularly, to a possible conformational rearrangement of the active site region. Nonetheless, it has to be noted that the substrate binding capacity of AcPDro is not significantly different from that shown by the human isoenzymes, as demonstrated by the K_m and K_i values, suggesting that the architecture of the active site is basically the same for all acylphosphatases studied so far. The most relevant difference between AcPDro and the Δ22 mutant concerns the apparent K_m and K_i values (up to 10-fold higher in the deletion mutant), suggesting that in the *D. melanogaster* enzyme the C-terminus tail might play a role in substrate binding.

The urea denaturation experiments demonstrate that the conformational stability of AcPDro is sharply reduced as compared to the human enzymes. A recent study performed on the human muscle enzyme showed that the human protein unfolds reversibly at pH 5.5 following a two-state model [27]. It can be observed that AcPDro at pH 7.0 behaves similarly to the human enzymes though its conformational stability is well below to that of the human enzymes and, in general, to that typically reported for globular proteins of similar size [28]. The removal of the 22 residues C-terminal tail does not determine a significant change of $\Delta G(H_2O)$, thus confirming that such amino acid appendix is not important for the three-dimensional structure stabilisation of the *D. melanogaster* enzyme. The strongly reduced conformational stability of the AcPDro as compared to the human enzymes still remains to be explained. A possible contribution to such conformational destabilisation could be due to the substitution of threonine 42, a conserved residue in all vertebrate acylphosphatases whose presence is critical for maintenance of the correct global enzyme conformation [13]. However, it seems unlikely to attribute such big change in $\Delta G(H_2O)$ exclusively to a conservative serine-threonine replacement: it is, therefore, necessary to hypothesise that other important contributions must be involved in the loss of stability of AcPDro. In any case, a further detailed structural analysis is required to clarify this aspect.

The high conservation of acylphosphatase amino acid sequences, observed in different vertebrate species, has suggested that the enzyme can tolerate only limited residue substitutions; the increased homology between the two isoenzymes found in more ancient vertebrates has also suggested possible gene duplication and a subsequent divergent evolution. Moreover, the ubiquitous presence in all vertebrate tissues of these enzymes implies their involvement in very important functions in cell. In this view we have looked for acylphosphatase presence in lower organisms. Previous observations of little acylphosphatase activity in *E. coli*, *Saccharomyces cerevisiae* and *Acrida bicolor* have been attributed to the action of acid phosphatases on acylphosphates [1]. The discovery of a *D. melanogaster* ORF coding for a small protein which displays acylphosphatase activity demonstrates that the enzyme is conserved during evolution also in non-vertebrate species. Furthermore, the comparison of the amino acid sequence of AcPDro to those of the vertebrate enzymes (Fig. 1) shows that the *D. melanogaster* enzyme is equally far from the muscle- and the common-type isoenzymes, providing an indication of gene duplication during evolution. Such hypothesis is supported by the putative phylogenetic tree prediction shown in Fig. 3 eliciting a cluster arrangement of the two groups of isoenzymes far apart from the *Drosophila* enzyme. The latter could be therefore considered as the product of a gene related to a common ancient precursor from which, late during phylogenesis, two branches of mutated genes originated through duplication and divergent evolution. Nevertheless, further investigation is required to definitely confirm such

hypothesis; in particular we cannot exclude the existence of another gene encoding AcP in the *D. melanogaster* genome, which has not been completely sequenced so far. The presence of acylphosphatase in *D. melanogaster* could open a new perspective concerning the role of this enzyme, suggesting a primary function in some basic biochemical processes: the duplication of the gene and the subsequent divergent evolution could have differentiated the function of the two isoenzymes in the higher organisms.

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