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Insights into acylphosphatase structure and catalytic mechanism

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Abstract. Acylphosphatase is one of the smallest enzymes known (about 98 amino acid residues). It is present in organs and tissues of vertebrate species as two isoenzymes sharing over 55% of sequence homology; these appear highly conserved in differing species. The two isoenzymes can be involved in a number of physiological processes, though their effective biological function is not still certain. The solution and crystal structures of different isoenzymes are known, revealing a close packed protein with a fold similar to that shown by other phosphate-binding proteins. The structural data, together with an extended site-directed mutagenesis investigation, led to the identification of the residues involved in enzyme catalysis. However, it appears unlikely that these residues are able to perform the full catalytic cycle: a substrate-assisted catalytic mechanism has therefore been proposed, in which the phosphate moiety of the substrate could act as a nucleophile activating the catalytic water molecule.
Key words. Acylphosphatase isoenzymes; acylphosphatase, catalytic mechanism; acylphosphatase, catalytic residues; acylphosphatase, structure.

Overview

Acylphosphatase (E.C. 3.6.1.7) is one of the smallest enzymes known (11,365 Da for the horse muscle form) [1]. It catalyses the hydrolysis of acylphosphates, compounds containing a carboxylphosphate bond such as 1,3-*bis*phosphoglycerate, carbamoylphosphate, succinylphosphate, acetylphosphate and β-aspartylphosphate [2–4]. These molecules play very important metabolic and physiological roles as intermediates in glycolysis, the tricarboxylic acid cycle, pyrimidine and urea biosynthesis and in the activity of membrane ion pumps. Unlike alkaline phosphatases and low M_r acidic phosphatases, the enzyme is unable to hydrolyse any phosphate ester or organic anhydride and is structurally different from alkaline phosphatase [5] and low M_r acidic phosphatases [6].

Acylphosphatase activity was first described in 1946 by Lipmann [7] and subsequently investigated, in the urea cycle studies, as the activity responsible for carbamoylphosphate hydrolysis [8, 9]. After the discovery of the carbamoylphosphatase activity in rat liver, similar activities were described in many tissues and in *E. coli* [10]; in addition, it was soon evident that the carbamoylphosphatase activity was due to the same enzyme responsible for the acetylphosphate hydrolysis previously described by Lipmann. Since that time, both carbamoylphosphatase and acetylphosphatase activities have been referred to as acylphosphatase.

Acylphosphatase is a cytosolic enzyme widely distributed in vertebrate species where it is found in many organs and tissues as two isoenzymes, named muscle type and erythrocyte (organ common) type, on the basis of the tissue in which each isoenzyme predominates or from which it was isolated for the first time. An acylphosphatase activity which does not correspond to any isoenzyme is also present in bacteria [10], yeast [11], and in seeds of *Vigna unguiculata* [12]. However, this activity is probably due to some acidic phosphatase rather than to acylphosphatase isoenzymes; in fact the genes for such enzymes have never been found in yeast or bacteria. The acylphosphatase activity found in mitochondria and in the sarcoplasmic reticulum of skeletal muscle is due to the presence of membrane ATPases, which are able to hydrolyse acylphosphates instead of ATP [13]. Recently, in addition to the cytosolic location, the enzyme has also been detected in the cell nucleus, where its presence depends on the cell cycle stage (unpublished results). In vertebrate skeletal muscle, the acylphosphatase activity content per gram of tissue sharply rises from fish to reptiles and birds; the rise is even more pronounced in mammals, suggesting a progressive increase of the enzyme importance during phylogenesis, mainly in warm-blood species. A correlation can be found between enzyme content in skeletal muscle and thermogenesis, suggesting a possible acylphosphatase involvement in thermoregulation.

Muscle acylphosphatase turnover has been investigated both in vivo and in cultured cells. Half lives of 6.8 days in the living muscle, 3 h 30 min in myoblasts, and 2 h 18 min in myotubes have been reported [14, 15]. The difference between these half lives could be due to different biological signals affecting protein expression and degradation in vivo and in cultured cells. The values reported for myoblasts and myotubes indicate

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that the acylphosphatase muscular isoenzyme can be considered a short-lived protein, though it does not contain any stretch particularly rich in PEST residues [15].

The two acylphosphatase isoenzymes share over 50% sequence homology, suggesting that their genes derive from a common ancestor by duplication and subsequent evolution, as confirmed by the increase in homology in phylogenetically more ancient organisms [16]. The amino acid sequence of each isoenzyme appears highly conserved in differing species; this finding suggests that the acylphosphatase structure can tolerate only limited substitutions, as would be expected in a very small protein (see 'Primary structure' section).

As previously indicated, limited information is currently available on the physiological role(s) of each acylphosphatase isoenzyme. The acylphosphatase physiological function(s) appears linked to the type of reaction catalysed and of substrate hydrolysed. Both carbamoylphosphatase and 1,3-*bis*phosphoglycerate phosphatase activity suggests an acylphosphatase regulatory role in glycolysis and pyrimidine biosynthesis [17]. As for carbamoylphosphatase activity, the enzyme could be involved in the control of the intracellular carbamoylphosphate levels in those cells lacking the urea cycle enzymes, thus avoiding the carbamoylation and subsequent alteration of the functional properties of a number of proteins [17, 18]. Acylphosphatase is also able to prevent histone acylation by 1,3-*bis*phosphoglycerate [19]. The action on 1,3-*bis*phosphoglycerate suggests that acylphosphatase isoenzymes could act as safety valves preventing the intracellular accumulation of such an intermediate. Acylphosphatase is able to stimulate the rate of retina glycolysis [2] and yeast fermentation and probably lowers the energetic yield of these processes, bypassing the phosphoglycerate kinase step through an uncoupling effect. Recently, an increase in the rate of ethanol production in *Saccharomyces cerevisiae* cells overexpressing a synthetic gene for muscle acylphosphatase has been shown [20]. The existence of a relation between glycolysis and acylphosphatase is supported by the increase in the content of the two isoenzymes in chicken muscle after hatching (when glycolysis is fully activated) [21] and further confirmed by the high acylphosphatase content in tissues, such as skeletal muscle, brain, testis, heart, and retina, where glycolysis is very active.

A lot of data have recently been reported indicating an acylphosphatase effect on membrane pumps [22–25]. Acylphosphatase is able to hydrolyse the β-aspartylphosphate formed during the action of both Ca^{2+}- and Na^+, K^+-ATPase of neuronal [4] and erythrocyte [23] plasma membranes, as well as cardiac [24] and skeletal muscle [4] sarcolemma. The erythrocyte isoenzyme shows a very high affinity ($K_m = 3.41$ nM) for the phosphorylated intermediate formed during the action of the erythrocyte membrane Ca^{2+}-ATPase [22]. High affinities for the intermediates formed by the action of the erythrocyte membrane sodium pump [23] and of the heart sarcolemma Na^+, K^+-pump [24] have also been reported, with K_m values of 147 nM and 69 nM, respectively. The addition of physiological levels of acylphosphatase to erythrocyte membrane preparations resulted in a significant calmodulin-independent increase in ATP hydrolysis accompanied by a decrease in both the Ca^{2+} transport rate and the ratio Ca^{2+}/ATP hydrolysed [22]. A similar uncoupling effect was found for the heart sarcolemma Ca^{2+} pump and for the Na^+, K^+-pump both in erythrocyte membrane [24] and in heart sarcolemma [23] in the presence of physiological amounts of either muscle or erythrocyte acylphosphatase. Using an inside out vesicle model system, it has been suggested that the acylphosphatase effect on the phosphoenzyme could occur at varying steps before and after the conformational changes encompassing Na^+ transport but before K^+ binding. Acylphosphatase could therefore modify the normal ordered reaction sequence and short-circuit the system [23]. The increase of phosphoenzyme hydrolysis rate and the consequent enhancement of the heart sarcolemmal Na^+, K^+-ATPase activity suggest a possible regulatory role of acylphosphatase isoenzymes in ion transport systems.

Changes in acylphosphatase cellular levels have been detected during erythrocyte ageing and in the differentiation of cultured myoblasts to myotubes [15]. In aged erythrocytes, the raise of enzyme content and hence of the uncoupling effect through membrane phosphorylated intermediate hydrolysis, is accompanied by a fall of the glycolytic pathway; these effects could contribute to a reduction of the energetic potential in these cells. A raise of acylphosphatase, as well as other muscle-specific protein content has been shown during myoblast differentiation. In the myoblast/myotube system, acylphosphatase expression can be considered an early event in cell differentiation; this finding, together with those relative to the ontogenic expression of the two isoenzymes, to the acylphosphatase effect on phosphoinositide pattern [1] and on the Ca^{2+} and other ion membrane transport systems, supports a possible role of acylphosphatase in cell differentiation. Nevertheless, it is not clear whether the raise of acylphosphatase content is one of the causes or the consequence of cell differentiation.

Acylphosphatase levels decrease significantly in muscles affected by varying types of myopathies [26] and increase in erythrocytes from hyperthyroid patients [27] as well as in erythrocytes and other cell types from triiodothyronine-treated rabbits [28]. Acylphosphatase reduction appears significantly correlated with Ca^{2+} activation percentage of sarcoplasmic reticulum Ca^{2+}, Mg^{2+}-ATPase, suggesting a link with enzyme action on membrane pumps. Acylphosphatase raise in hyperthy-

roid patients correlates to biochemical parameters of thyroid status, particularly thyroid hormone levels, and it is lowered when patients are treated with pharmacological therapy [27]. T3-induced acylphosphatase expression in erythro-leukemia cells has recently been shown [29]. This finding suggests that the rise in acylphosphatase activity in hyperthyroid patients can be considered as the consequence of an increase in enzyme biosynthesis in erythroid cells rather than of a direct action on the enzyme itself; it also stresses a possible clinical use for the acylphosphatase assay in diagnosis of hyperthyroidism. Recently, a rise in acylphosphatase erythrocyte isoenzyme content has been found in cultured skin fibroblasts from patients affected by early-onset familial Alzheimer's disease [30]. This could explain the calcium-dependent biochemical alterations previously described in Alzheimer's disease fibroblasts.

The role of the muscular isoenzyme in *Xenopus laevis* oocyte maturation has been investigated by microinjecting acylphosphatase into cells physiologically arrested in the G2/M phase border of the first meiotic division and previously treated with either progesterone or recombinant ras-p21, or untreated [31, 32]. The results indicated that acylphosphatase synergizes with progesterone in inducing oocyte maturation but blocks p21-stimulated maturation. These effects have been considered as the consequence of the alteration of the intracellular Ca^{2+} (and possibly other ion) levels following acylphosphatase interaction with membrane ion pumps.

Despite the number of findings currently available concerning acylphosphatase involvement in several biochemical and physiological processes, the true biological role of acylphosphatase is still debated.

This review deals with the currently known structural and catalytic features of acylphosphatase isoenzymes. The structure, folding, and catalytic residues of acylphosphatase isoenzymes are quite characterized; on the basis of these findings, a convincing model of the enzyme catalytic mechanism is emerging (see 'Active site structure and enzyme catalytic mechanism' section). The data at present available on this topic and those that will be obtained in the near future from the use of negative dominants, will open perspectives for a better understanding of the physiological function(s) of the enzyme.

Purification and primary structure

Acylphosphatase muscular isoenzyme was first purified from horse muscle, heart and liver and from bovine brain [33–35] as well as from turkey, human, guinea pig, rabbit, pig, chicken, ox, duck and rat skeletal muscle [1, 14, 26, 36–38], using either a standard or an immunoaffinity chromatography-based purification procedure. In 1986, the erythrocyte isoenzyme was purified to homogeneity from human erythrocytes and characterized [39]. The same isoenzyme has more recently been purified from turkey and chicken muscle, bovine and porcine testis, horse brain and bovine erythrocytes [1, 37, 40, 41]. The two isoenzymes differ from each other in amino acid composition, and N-, and C-terminal residues. The main physicochemical parameters of the muscle isoenzyme, comprising sedimentation coefficient, diffusion constant and partial specific volume, have been determined, together with an isoelectric point of about 11.4 [33]. The muscular and erythrocyte isoenzymes from porcine testis show isoelectric points of 10.6 and 8.3, respectively [40].

Acylphosphatase was first sequenced in 1980, when the horse muscle isoenzyme primary structure was reported [42]. In the following years many other mammal and avian muscle acylphosphatase sequences have been reported (fig. 1) [1, 14, 38, 43–45]. In general, the protein is composed of 98 amino acid residues (M_r 11,365) and is acetylated at the N-terminus. It lacks histidine residues and contains a sole cysteine residue; the latter is present in the reduced (-SH) form in vivo, though the enzyme is also purified both as glutathione-mixed disulphide and as an S-S dimer. These enzyme forms have a reduced specific activity and a higher stability and are considered artefacts arising from the enzyme purification conditions [46]. Figure 1 shows that muscle acylphosphatase is a highly conserved enzyme and that the most variable part of the molecule is located at the N-terminus; this region can also display insertions (four residues in avian enzymes) or deletions (two residues in the rat enzyme). Furthermore, the highly variable NH_2 tail appears to be the most unstructured and mobile part of the molecule (see 'Three-dimensional structure and stability' section).

In 1986, the primary structure of the human erythrocyte isoenzyme was reported [16]; later, the same isoenzyme was sequenced from other sources [41, 47, 48]. The enzyme sequence appears highly homologous (about 56%) to that of the human muscle isoenzyme previously reported. It is composed of 98 amino acid residues with the N-terminus acetylated and the terminal residues present differ from those of the muscle isoenzymes. In addition, the erythrocyte isoenzyme contains histidine residues but lacks cysteine and hence, unlike the muscular isoenzyme, has not been purified in differing molecular forms. Again unlike the muscular one, the erythrocyte isoenzyme is composed of 98 amino acid residues both in mammals and birds (fig. 1). Only the testis enzyme is present in two molecular forms found in similar amounts, one containing two additional residues at the N-terminus. Both forms are N-acetylated and hence the shorter cannot be considered a product of the partial proteolytic degradation of the longer one [41, 47]; it is possible that both forms originate from post-translational processing. The significance of the existence of differing acylphosphatase isoenzymes and

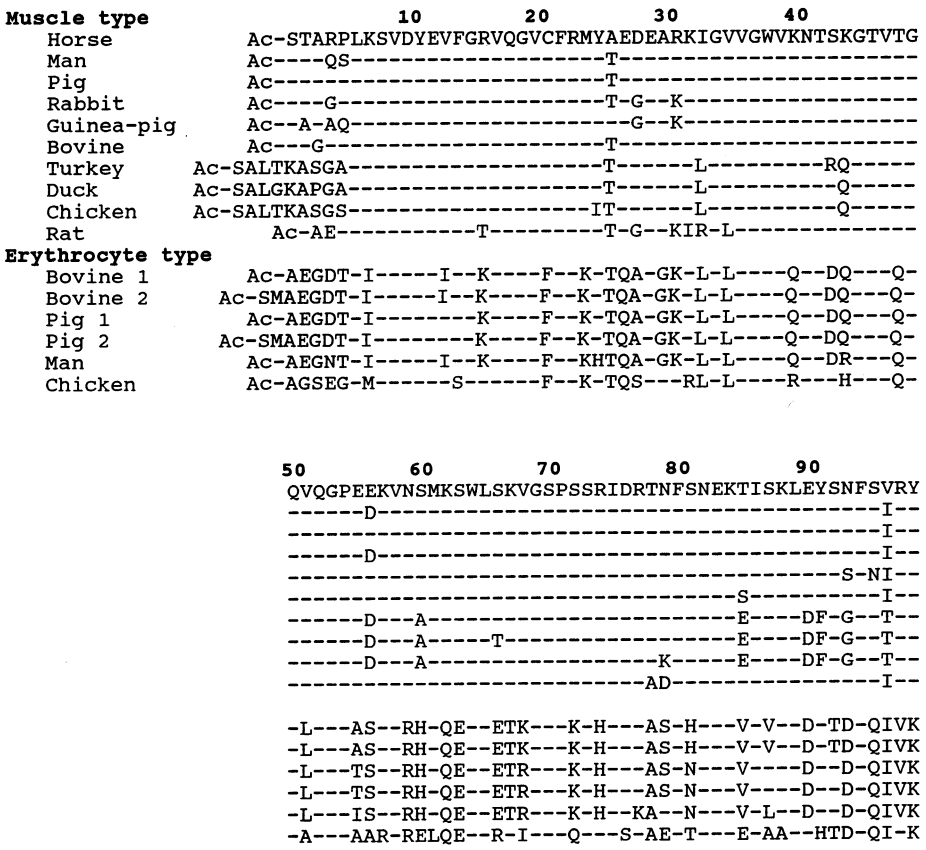

Figure 1. Alignment of the amino acid sequences of the muscular and erythrocyte acylphosphatase isoenzymes.

molecular forms in the same tissues needs further investigation; a possible explanation is that they could be differently regulated in cells or act on different targets.

The homology between muscular and erythrocyte isoenzymes suggests that their genes are derived from a common ancestor by duplication and subsequent evolution. This hypothesis is supported by the finding that the degree of homology between the two isoenzymes progressively rises in the more phylogenetically ancient species. In fact, from the amino acid sequence analysis of the mammal and avian enzymes and those from a bony fish (Cappugi, G., personal communication), an average homology of about 57%, 62%, and 69%, respectively, can be calculated. From these data, and assuming a constant mutation rate of the acylphosphatase genes, the time of gene duplication can tentatively be dated to about 500 million years ago.

Kinetic properties and chemical modifications

Acylphosphatase isoenzymes are able to catalyse the in vitro hydrolysis of all acylphosphates so far tested, with specific activities ranging from about 10 to 10,000 (UI/mg of protein) and substrate affinities of 0.1–1 mM depending upon isoenzyme type and molecular form [1]; in addition, as underlined in the Overview, both isoenzymes are able to dephosphorylate the phosphorylated intermediate formed by the action of membrane ion pumps, with affinities falling in the nanomolar range. The erythrocyte isoenzyme and the reduced (-SH) form of the muscular isoenzyme show very similar in vitro affinities and specific activities in the presence of benzoylphosphate, a synthetic substrate used for the routine assay of enzyme activity, whereas the mixed disulphide with glutathione and the S-S dimer of the muscular isoenzyme show reduced affinities and specific activities [46]. None of the isoenzymes is able to hydrolyse phosphoester bonds or carboxylphosphate bonds in which a substituted phosphate group is involved; in addition, both isoenzymes are able to bind substrates only when the phosphate group is present in the dianionic form [49]. Thus, compounds such as phosphocreatine, ATP, acetyl-AMP, pyrophosphate, phosphoenolpyruvate and phosphate esters are not hydrolysed [50, 51]. Moreover, both isoenzymes are inactive at acidic pH values, when the phosphate moiety of the substrate is present in the monoanionic form.

Both acylphosphatase isoenzymes are competitively inhibited by the reaction product, inorganic phosphate and, less specifically and less efficiently, by other inorganic anions such as chloride and sulphate and by

phosphorylated compounds [3]. The acylphosphatase muscular isoenzyme is also inhibited by heavy metal cations such as Hg^{2+} and Ag^{+} (unpublished results), as well as by orotate and by pyridoxal 5′-phosphate [1, 52]. The latter appears to inhibit the enzyme reversibly by forming a Schiff base with the side chain of one (or more) lysine residue(s); since pyridoxal alone does not inhibit the enzyme, the phosphate moiety of pyridoxal 5′-phosphate appears necessary to position the reactive group properly, indicating the probable presence of a lysine residue in or near the active site. In addition to pyridoxal 5′-phosphate, other agents have been used to study the residues involved in the catalytic mechanism by chemical modifications of enzyme-specific residues. In particular, phenylglyoxal, a specific reagent for arginine side-chains, inactivates both erythrocyte and muscular acylphosphatase in a time- and concentration-dependent manner; such inactivation is prevented by the presence of inorganic phosphate, indicating that it proceeds via chemical modification of at least one arginine residue located in or near the active site [1, 53]. Similarly, the Woodward's reagent K, a compound generally used for chemical, though not strictly specific, modification of protein carboxyl groups, inactivates the erythrocyte isoenzyme in a time- and concentration-dependent manner; this inactivation is also prevented by the presence of inorganic phosphate [54].

Three-dimensional structure

The first approach to the study of the three-dimensional structure of acylphosphatase was carried out by circular dichroism and prediction studies based on the Chou-Fasman algorithm [1]. Such an investigation, performed on the horse muscle isoenzyme, indicated the presence of both α-helix and β-sheet secondary structure elements. At around the same time the crystallisation of the protein was attempted and small plated crystals, unfortunately not suitable for a high-resolution X-ray diffraction study, were grown. The three-dimensional solution structure of the muscle isoenzyme has been determined by ^{1}H NMR spectroscopy [55–60]. According to this study, acylphosphatase is a globular α/β protein formed by a five-stranded antiparallel twisted β-sheet facing two antiparallel α-helices. The helices, running parallel to the sheet, protect one side of the sheet while the other side is exposed to the solvent. An extended interaction surface between the sheet and the helices provides the hydrophobic core of the enzyme. The secondary structural elements in muscle acylphosphatase can be considered as two intercalating $\beta\alpha\beta$ subunits arranged in a 4-1-3-2 β-strand topology. A similar fold has been also observed in ferrodoxin [61], the activation domain of procarboxypeptidase B [62, 63], the RNA binding domain of the small nuclear ribonucleoproteins A and C [64, 65], the C-terminal domain of ribosomal proteins L7/L12 [66] and L30 [67], and the bacterial mercuric ion binding protein (MerP) [68]. The histidine-containing phosphocarrier protein (HPr) shows a similar fold despite a different β-strand topology [69, 70].

Since most of the above mentioned proteins are involved in phosphate binding and do not share any significant sequence homology, it has been suggested that a common phosphate binding motif could exist [71]. Such a motif should be located at the N-terminus of an α-helix displaying basic residues in this region, hence favouring the binding of the phosphate anion. However, ^{1}H NMR data, though essential in describing the low resolution three-dimensional structure in solution, provided little information about the location of the active site. Additional experiments performed using $[Cr(CN)_6]^{3-}$, an acylphosphatase competitive inhibitor, as a relaxation probe indicated the possible location of the enzyme active site near the C-terminus [72]. No information was obtained about the identity of the residues involved in either catalysis or substrate binding.

Recently, acylphosphatase erythrocyte isoenzyme crystals were grown in 25 mM acetate buffer containing 30% PEG, 0.2 M ammonium sulphate at pH 3.5 [73]. Such crystals, approximately $0.1 \times 0.3 \times 0.3$ mm, belonging to space group C2, were suitable for a high-resolution X-ray diffraction study. Very recently, the crystal structure of the acylphosphatase erythrocyte isoenzyme has been solved at 1.8 Å resolution with an R factor of 17.0% [74]. The overall structure is very similar to that of the muscular isoenzyme previously determined by ^{1}H NMR; the erythrocyte isoenzyme is a very compact globular α/β protein composed of a five-stranded antiparallel twisted β-sheet facing two antiparallel α-helices (fig. 2). After careful comparison, only minor and local differences between the structures of the two isoenzymes have been demonstrated. These differences mostly involve the loop regions (mainly the 14-21 loop) and have been attributed to the presence of a sulphate and a chloride ion in the active site of the X-ray structure [74]. In fact, the residues of those loops appear quite well conserved and hence not likely to be the cause of the conformational differences found between the structure of the muscle isoenzyme in solution and that of the erythrocyte isoenzyme in the crystalline state.

A major feature arising from the crystal structure of the erythrocyte acylphosphatase is the full structural characterisation of the loops, particularly those involving residues 14–21, 42–45, and 68–74. In the ^{1}H NMR structure, these loops were not deeply investigated due to their mobility in solution [72]. In particular, the crystal structure has shown the presence of a sulphate and a chloride ion near the Arg 23 and Asn 41 side-chains, making extensive contacts to the last residues of

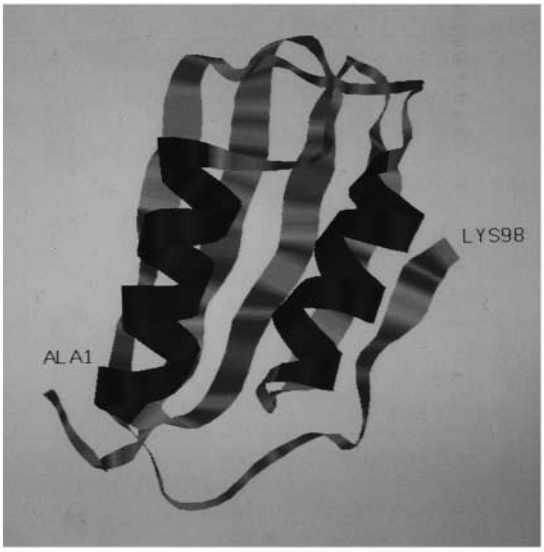

Figure 2. Outline of the three-dimensional structure of the acylphosphatase erythrocyte isoenzyme as determined by X-ray diffraction.

the loop 14–21 (fig. 3); these ions were present in the crystallisation buffers, and their presence in the enzyme structure together with the existence of crystal contacts in the crystal lattice could be responsible for a little distortion on the active site. The detailed description of the environment surrounding the two anions in the enzyme crystal structure allowed the acylphosphatase active centre to be identified; these data, together with the results obtained by mutagenesis experiments (see later), led to a proposal for the enzyme catalytic mechanism (see below).

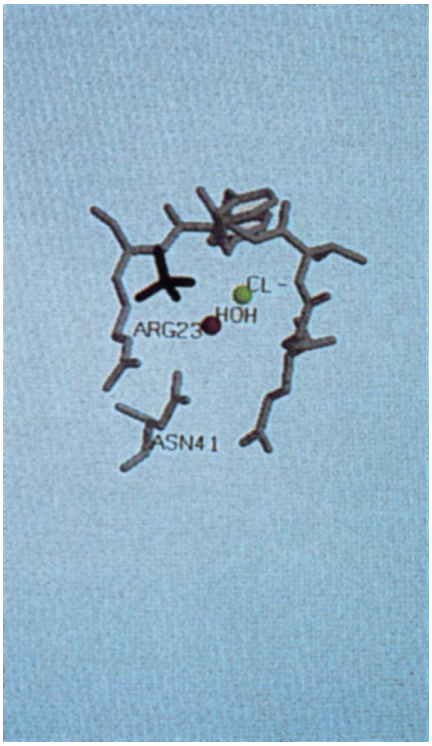

Figure 3. Schematic diagram illustrating the acylphosphatase active site residues as indicated by the erythrocyte isoenzyme crystal structure. The darker structure is the sulphate ion, near the catalytic water molecule and the chloride ion.

The description of the acylphosphatase erythrocyte isoenzyme crystal structure reveals a surprising similarity between its phosphate binding loop and that previously described in the low molecular weight phosphotyrosine protein phosphatase (low M_r PTPase). In particular, when the phosphate binding loops of the two structures are superimposed, critical catalytic residues such as Arg23 and Asn41 in acylphosphatase occupy positions strictly corresponding to those occupied by Arg18 and Asp129 in the PTPase [74]. This evidence, together with the correspondence of the phosphate-binding site in acylphosphatase and in other phosphate-binding proteins such as HPr, supports the structural conservation of catalytic regions in different enzymes involved in the biochemical utilisation of phosphate-containing substrates.

Folding and stability

The stability of the muscle isoenzyme has been investigated by different techniques. Most of the initial studies concerning acylphosphatase stability were carried out on the three different forms in which the enzyme was purified (mixed-disulphide with glutathione, S-S dimer and reduced, -SH, form) using the enzymatic activity as a probe for the native conformational state [1, 75, 76]. Such experiments, mainly performed to evaluate irreversible denaturation, indicated that the enzyme is considerably heat-resistant, especially in the glutathione mixed-disulphide form and S-S dimer; for the former, an activation energy for the denaturation process of about 9500 cal/mol was calculated at 25 °C [75]. In addition, the Arrhenius plot shows a biphasic character with a transition temperature at 65 °C, when the activation energy of the denaturation process drops to about 970 cal/mol [75]. More recently, equilibrium unfolding and stability of horse muscle acylphosphatase have been investigated by circular dichroism and ^{1}H NMR [77]. The experiments were carried out under varying pH, temperature and urea concentration conditions and demonstrated that the enzyme unfolds cooperatively and reversibly following a two-state transition in all cases. No stable unfolding intermediates were isolated under those experimental conditions. The same study indicated that unfolded acylphosphatase maintains little residual structure [77]. The refolding kinetic is currently being investigated by stopped-flow circular dichroism, fluorescence and ^{1}H NMR spectroscopy; preliminary results indicate that the muscle enzyme refolds cooperatively from a guanidinium chloride-denatured state (data not published). Additional data on the importance of single residues or secondary structural elements

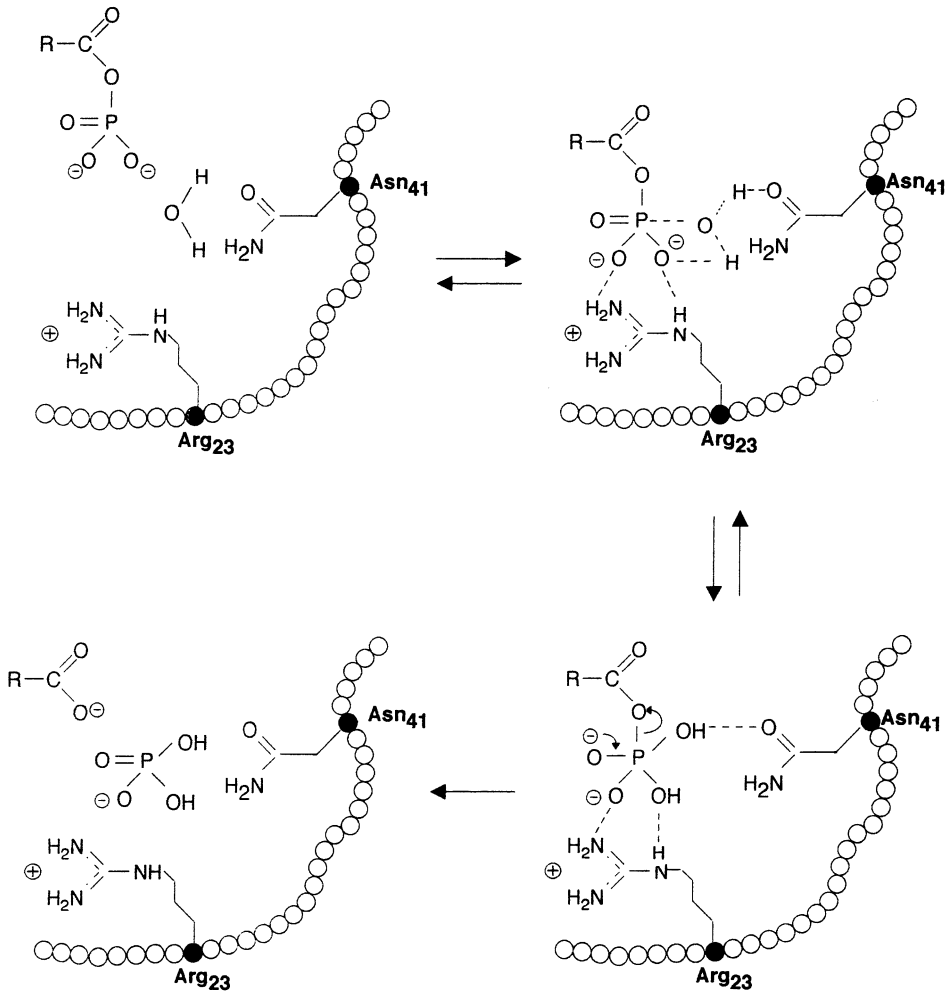

Figure 4. Proposed catalytic mechanism of acylphosphatase.

in the overall structure stabilisation have been provided by mutagenesis studies (see later).

Muscular isoenzyme mutagenesis

The information on acylphosphatase structure-function relationships has been greatly enhanced by using *E. coli* cells engineered with a chemically synthesized gene coding for the human muscle isoenzyme [78]. High expression levels were achieved and the gene product purified and characterised. Comparison with the natural wild-type enzyme showed the absence of any significant structural and kinetic difference between the two proteins, except the lack of N-acetylation in the recombinant form. Recently, a different expression system has been developed and used [79], giving rise to a more rapid enzyme purification procedure. A cDNA coding for the human erythrocyte isoenzyme has also been isolated and cloned [80]. On the basis of the available structural data and taking into account previous suggestions on the enzyme catalytic mechanism based upon a kinetic analysis [49], a site-directed mutagenesis investigation of acylphosphatase isoenzymes was started. This investigation aimed to describe the role and importance of specific residues in enzyme catalysis and function. Table 1 reports the main kinetic parameters calculated for the mutants investigated so far.

Arginine 23

The presence of a residue with $pK_a > 12.0$ involved in substrate binding was suggested 25 years ago by Satchell et al. [49]. These authors tentatively identified this residue as an arginine due to the absence, in the enzyme structure, of any metal ion. Later, inactivation studies performed on acylphosphatase chemically modified by phenylglyoxal, a site-specific reagent for arginine residues (see 'Kinetic properties and chemical modifications' section), confirmed this hypothesis [1, 53]. Evidence of structural similarities between unrelated phosphate-binding proteins led Swindells et al. to hypothesize the involvement of an arginine residue located at the N-terminal edge of an α-helix as a phosphate-binding residue [71]. In the case of acylphosphatase,

such a residue could be arginine 23, one of the four arginine residues highly conserved in all acylphosphatases sequenced. The actual role of this residue in substrate binding has been shown conclusively through the preparation, purification, and characterization of three muscular acylphosphatase mutants, where arginine 23 was replaced by either glutamine, histidine or lysine [81]. All mutants were almost completely devoid of catalytic activity and were unable to bind inorganic phosphate. Nevertheless, the mutants maintained a native-like global fold, indicating that arginine 23 is indeed a residue involved in substrate binding rather than in structural stabilisation.

C- and N-terminus regions

Acylphosphatase C- and N-terminus regions were investigated to evaluate the effect of protein shortening on the enzyme biological activity. In fact, a previous study performed on an acylphosphatase with an enzymatically truncated C-terminus indicated that the last two residues were essential for enzyme activity [82]. The possibility of a C-terminal carboxylate group involvement in the catalytic mechanism, as suggested by previous kinetic and structural studies [49, 72, and unpublished data], was also evaluated. Different muscle acylphosphatase mutants were thus prepared and studied. Arginine 97 or tyrosine 98 were replaced by a glutamine residue; the first six residues were removed in an N-terminus deletion mutant; finally, the C-terminus was modified by preparing both deletion mutants (lacking the two or three C-terminal residues) and insertion mutants, containing two or four additional residues at the C-terminus [83, 84]. This mutagenesis study showed that the acylphosphatase N-terminus is neither involved in the catalytic mechanism nor in enzyme stabilisation. Contrary to previous suggestions, the study also demonstrated that the C-terminal region is not directly involved in catalysis but it probably plays a critical role in active site structural stabilisation. No evidence was provided for terminal carboxylate group involvement as a nucleophile in the catalytic mechanism.

Cysteine 21

Cysteine 21 is an invariant residue in all muscle acylphosphatase isoenzymes sequenced so far, whereas in the erythrocyte isoenzymes it is replaced by a phenylalanine, suggesting that this residue is marginally important in the catalytic mechanism. Significant kinetic differences between the three different isoforms isolated from muscle were observed. In particular, the results demonstrating that the muscular isoenzyme in vivo possesses the cysteine residues in the reduced (-SH) form [46] suggested a possible involvement of such residue in enzyme catalysis. The kinetic and structural features of two mutants in which cysteine 21 was substituted by either alanine or serine [85] were comparable to those of the wild-type enzyme, thus excluding a direct involvement of this residue in catalysis. However, the spatial proximity of cysteine 21 to arginine 23 and the full exposure to the solvent of the corresponding phenylalanine residue present in the erythrocyte isoenzyme, suggest a role of the residue at position 21 in substrate recognition and binding [74].

Asparagine 41, threonine 42 and threonine 46

Asparagine 41, threonine 42 and threonine 46 are invariant residues in all acylphosphatase isoenzymes so far sequenced. The solution structure of the muscle isoenzyme indicates that these residues are positioned in the neighbourhood of arginine 23 [60]. Any of them could therefore be involved in binding and orienting the catalytic water molecule [49]. The individual roles of these residues have been determined by site-directed mutagenesis [86]. The kinetic and structural characterisation of acylphosphatase mutants where asparagine 41 was substituted by alanine, serine or glutamine showed the critical importance of this residue for enzymatic activity. In fact, all mutations at position 41 resulted in an almost complete inactivation of the enzyme with no effects on substrate binding capacity, though the overall mutant folds were not significantly different from that of the wild-type protein. Asn41 mutants appear therefore to be good candidates for use as negative dominants in cell biology experiments centred on the investigation of the acylphosphatase physiological function(s). The same study showed that mutation to alanine of either Thr42 or Thr46 did not inactivate the enzyme, allowing the direct participation of such residues in catalysis to be excluded. However, the stabilities of both 42 and 46 mutants were considerably altered, as compared to the wild-type enzyme, indicating an involvement of these residues, which are part of the 42–45 loop, in the enzyme three-dimensional structure stabilisation.

Active site structure and enzyme catalytic mechanism

The mutagenesis experiments performed on the acylphosphatase muscular isoenzyme led to identification of two catalytic residues: Arg23, which appears to be the main phosphate binding site, and Asn41, presumably involved in binding and orienting the catalytic water molecule. The presence of such residues had been predicted by the kinetic study of Satchell et al. on muscle acylphosphatase [49]. The same study also claimed the participation, in the catalytic mechanism of a protonated group with $pK_a = 11.0$, tentatively identified with the amino group of a Lys residue. However, mutagenesis experiments performed on both acylphosphatase isoenzymes indicated that no Lys (and no acidic)

Table 1. Main kinetic properties of mutated acylphosphatases[a].

Enzyme	pH optimum	Specific activity[b]	K_m (mM)[c]	K_i or K_d (mM)[d]	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild-type	4.8–5.8	6500–7500	0.36	0.75	3400–3920
R23Q	n.d.	4.5	n.d.	n.d.	n.d.
R23H	n.d.	5.5	n.d.	n.d.	n.d.
R23K	n.d.	15.4	n.d.	n.d.	n.d.
C21A	4.5–5.5	4300	0.40	0.90	2040
C21S	4.7–5.6	5900	0.80	0.70	1400
R97Q	3.7–4.4	4700	1.14	1.79	780
Y98Q	5.0–5.9	3600	0.41	1.17	1670
T42A	4.7–5.7	4700	0.70	1.17	1270
T46A	4.8–5.8	5000	0.66	1.47	1430
N41A	4.9–5.9	10	0.30	0.14	5.6
N41S	4.9–5.9	10	0.23	0.19	8.0
N41Q	4.9–5.9	3	0.55	1.51	1.0
Δ6	4.7–5.9	2500	0.55	1.19	860
Δ + 2	4.8–5.8	5800	0.37	0.77	3000
Δ + 4	4.6–5.6	5100	0.39	0.88	2480
Δ − 2	3.8–4.8	480	0.82	2.85	110
Δ − 3	4.0–5.0	280	1.40	4.70	38

[a]Data from 81, 83–86], [b]units/mg of protein, [c]using benzoylphosphate as substrate, [d]for inorganic phosphate.

residues are essential for enzyme catalysis [54 and unpublished results], indicating the absence of any other ionizable catalytic residue in the active site.

Recently, the crystal structure of the common-type isoenzyme has been determined at 1.8 Å resolution [74] (see also 'Three-dimensional structure' section). In the structure, the enzyme is complexed with a sulphate and a chloride ion; these occupy the active site, which appears as a shallow groove on the enzyme surface formed by the 14–21, 42–45, and 68–74 loops. The presence of the chloride ion, presumably an artefact due to crystal packing, pushes the sulphate ion out of the theoretically calculated position in the active site, in which the sulphate ion would be in close contact with Arg23 (fig. 3). The crystal structure also shows that the Arg23 side chain is near Asn41, which in turn binds a water molecule. Such a water molecule is in a suitable position for acting as a nucleophile when appropriately activated by deprotonation. Unfortunately, the crystal structure does not reveal any possible water-activating residue in the active centre region.

These results describe accurately the structure of the erythrocyte acylphosphatase active site; this can reasonably be considered strictly conserved in the muscular isoenzyme as well, raising the question of how the water molecule in the active centre is activated to serve as a nucleophile in the catalytic mechanism. In fact, it is unlikely to figure the Asn41 side chain functioning as water activator, but, on the other hand, no other group suitable for such an activation is present in the acylphosphatase active site nor has one been identified through mutagenesis experiments. A possible water activation, well supported by the crystal structure, could be through the phosphate ion acting as proton acceptor in a substrate-assisted catalytic mechanism. Such a mechanism has recently been proposed for GTP hydrolysis catalysed by $p21^{ras}$ and other GTP binding proteins [87–88]. According to this hypothesis (fig. 4), the water molecule bound to Asn41 would be activated by the protonation of the phosphate group present in the substrate molecule. The hydroxyl anion so generated would then bind the phosphorus atom producing a pentacoordinate intermediate, which could undergo decomposition with release of the leaving group and inorganic phosphate. However, such an intriguing hypothesis needs further experimental evidence to be fully accepted. In particular, a detailed kinetic analysis of the enzyme is needed, together with structural data from acylphosphatase mutated in the active centre.

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