Looking for Residues Involved in the Muscle Acylphosphatase Catalytic Mechanism and Structural Stabilization: Role of Asn41, Thr42, and Thr46[†]

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ABSTRACT: Asn41, Thr42, and Thr46 are invariant residues in both muscle and erythrocyte acylphosphatases isolated so far. Horse muscle acylphosphatase solution structure suggests their close spatial relationship to Arg23, the main substrate binding site. The catalytic and structural role of such residues, as well as their influence on muscle acylphosphatase stability, was investigated by preparing several gene mutants (Thr42Ala, Thr46Ala, Asn41Ala, Asn41Ser, and Asn41Gln) by oligonucleotide-directed mutagenesis. The mutated genes were cloned and expressed in *Escherichia coli*, and the mutant enzymes were purified by affinity chromatography and investigated as compared to the wild-type enzyme. The specific activity and substrate affinity of Thr42 and Thr46 mutants were not significantly affected. On the contrary, Asn41 mutants showed a residual negligible activity (about 0.05−0.15% as compared to wild-type enzyme), though maintaining an unchanged binding capability of both substrate and inorganic phosphate, an enzyme competitive inhibitor. According to the ^{1}H nuclear magnetic resonance spectroscopy and circular dichroism results, all mutants elicited well-constrained native-like secondary and tertiary structures. Thermodynamic parameters, as calculated from circular dichroism data, demonstrated a significantly decreased stability of the Thr42 mutant under increasing temperatures and urea concentrations. The reported results strongly support a direct participation of Asn41 to the enzyme catalytic mechanism, indicating that Asn41 mutants may well represent a useful tool for the investigation of the enzyme physiological function by the negative dominant approach.

Acylphosphatase (EC 3.6.1.7) is a very basic, 98 amino acid residue protein (MW 11 365, horse muscle enzyme) which catalyzes the hydrolysis of the carboxyl phosphate bond present in either synthetic and physiologically important molecules (Stefani & Ramponi, 1995). Among acylphosphatase substrates are acetyl phosphate, 1,3-bisphosphoglycerate, carbamoyl phosphate, succinyl phosphate, and the β-aspartyl phosphate intermediate formed during the action of membrane ion pumps (Ramponi, 1975; Stefani et al., 1981; Nediani et al., 1995). The enzyme elicits a strict specificity for carboxyl phosphate bond hydrolysis, since it appears inactive on phosphate monoesters, inorganic pyrophosphate, and phosphorylated nucleosides. In addition, the presence of the substrate in the double-deprotonated form seems to be essential for catalysis (Satchell et al., 1972).

The enzyme is widely distributed in vertebrate tissues in the form of two *N*-acetylated isoenzymes sharing over 50% sequence homology. The genes encoding the two isoenzymes are therefore likely to derive from a common ancestor by duplication and subsequent evolution (Liguri et al., 1986; Fujita et al., 1987; Mizuno et al., 1990). The two acylphosphatase isoenzymes are generally named muscle-type and erythrocyte-type (organ common-type) depending on the tissue where they were first purified or where they are particularly abundant. Muscle acylphosphatase isolated from several vertebrate species appears remarkably conserved (Pazzagli et al., 1993).

The three-dimensional structure in solution of the muscle isoenzyme has been determined by ^{1}H nuclear magnetic resonance (NMR)[1] spectroscopy (Saudek et al., 1989; Pastore et al., 1992). The enzyme is a closely packed α/β protein composed by two $\beta\alpha\beta$ motifs intercalated into each other, forming an antiparallel β-sheet. This particular fold has been found in a few other proteins, such as the bacterial histidine-containing phosphocarrier protein (HPr), the bacterial mercuric ion binding protein (MerP), the RNA binding domain of the U1 small nuclear ribonucleoprotein A, the DNA binding domain of the papillomavirus-1 E2 (BPV E2), the RNA binding domain of hnRNP C, and the activation domain of the pancreatic procarboxypeptidase (Nagai et al., 1990; Coll et al., 1991; Vendrell et al., 1991; Hegde et al., 1992; Wittekind et al., 1992; Eriksson et al., 1993; Van Nuland et al., 1994).

Acylphosphatase is supposed to interfere with glycolysis by enhancing the glycolytic flux and the rate of fermentation (Ramponi et al., 1989). Recently, a role has been proposed for the enzyme in the regulation of plasmamembrane ion pumps (Nassi et al., 1991, 1993; Nediani et al., 1995) and

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[1] Abbreviations: Asn41Ala, asparagine 41 to alanine mutant; Asn41Gln, asparagine 41 to glutamine mutant; Asn41Ser, asparagine 41 to serine mutant; Thr42Ala, threonine 42 to alanine mutant; Thr46Ala, threonine 46 to alanine mutant; NMR, nuclear magnetic resonance; CD, circular dichroism; CED, cyanoethyldeoxy; IPTG, isopropyl thiogalactoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; USE, unique site elimination.

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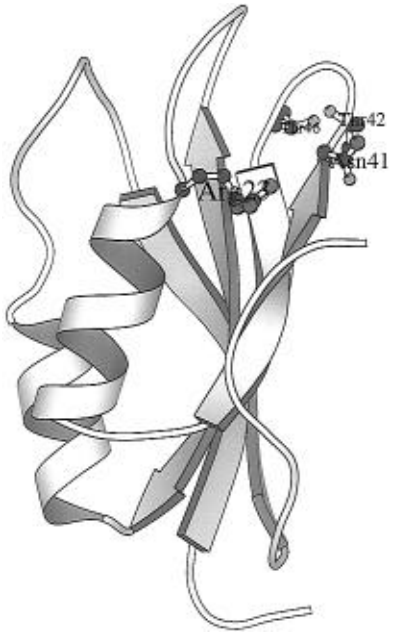

FIGURE 1: Outline of the three-dimensional solution structure of horse muscle acylphosphatase drawn using Molscript (Kraulis, 1991). Arg23, Asn41, Thr42, and Thr46 side chains are also indicated. The definition of secondary structure has an uncertainty of ±1 residue.

the related intracellular ion levels (Dolfi et al., 1993; Liguri et al., 1994). Despite these results, the physiological functions of both isoenzymes are still not completely understood.

Limited information is available concerning the acylphosphatase catalytic mechanism (Satchell et al., 1972). Oligonucleotide-directed mutagenesis experiments have recently indicated Arg23 as the main phosphate binding site in the muscular isoenzyme (Taddei et al., 1994a), confirming previous suggestions (Swindells et al., 1993). Arg23 is an invariant residue also in the erythrocyte isoenzyme, and therefore it can be assumed to perform the same function in both isoenzymes.

Asn41, Thr42, and Thr46 are among the invariant residues in both isoenzymes isolated so far (Pazzagli et al., 1993). These residues lie in close spatial proximity to Arg23 (Pastore et al., 1992), as shown in Figure 1. Any of them could therefore be involved in binding and orienting the water molecule responsible for O−P bond breakdown in the enzyme−substrate complex during catalysis as previously suggested (Satchell et al., 1972).

Looking for a completely inactive enzyme mutant still capable of substrate binding, we prepared five mutants (Asn41Ala, Asn41Ser, Asn41Gln, Thr42Ala, and Thr46Ala) of the muscular form by using the same oligonucleotide-directed mutagenesis approach. All mutants were investigated for kinetic and structural behavior as compared to the wild-type enzyme. The identification of such inactive mutants might provide important insights into the catalytic mechanism and the active site residues; it could also represent a useful tool for studying enzyme functions in cultured cells by the negative mutant approach.

MATERIALS AND METHODS

Materials. Benzoyl phosphate was synthesized as previously described (Camici et al., 1976) and freshly dissolved before use. Restriction and modification enzymes and isopropyl thiogalactoside (IPTG) were from Promega. The specific polyclonal anti-recombinant human muscle acylphosphatase antibodies used in the Western blotting were obtained as previously described (Berti et al., 1982). Horseradish peroxidase-linked mouse anti-rabbit Ig was obtained from Bio-Rad. [α-^{32}P]ATP (800 Ci/mmol) and [^{32}P]P_i (8500 Ci/mmol) were from New England Nuclear. Sequenase was from USB. pGEX-2T was from Pharmacia. The pGEX-AP plasmid is a derivative of the pGEX-2T vector harboring a chemically synthesized gene coding for human muscle acylphosphatase; it was prepared as previously described (Modesti et al., 1993). Plasmid propagation and recombinant fusion protein expression were achieved by using the DH5α and TB1 bacterial strains. Oligonucleotides harboring the mutated codons were synthesized using a Beckman System 200 A DNA synthesizer, by using the CED−phosphoramidite method. Glutathione, human thrombin, and glutathione−agarose affinity gel were from Sigma. D_2O and acetic acid-d_4 were from Merck (Darmstadt). All other reagents were analytical grade or the best commercially available.

Site-Directed Mutagenesis. Asn41 mutants to Ala, Gln, and Ser (Asn41Ala, Asn41Gln, and Asn41Ser, respectively) and Thr42 and Thr46 mutants to Ala (Thr42Ala and Thr46Ala, respectively) were obtained by oligonucleotide-directed mutagenesis using a USE mutagenesis kit based upon the unique site elimination (USE) method developed by Deng and Nickoloff (1992). Five target primers harboring the desired mutated codons were synthesized using the CED−phosphoramidite method. The target mutageneic primers were complementary to the gene coding for acylphosphatase and carried the desired mutations: AAC (Asn) to GCC (Ala), to CAG (Gln), and to AGC (Ser), respectively, and ACC (Thr) to GCC (Ala) (Table 1). The method utilizes the target mutagenic primers and a selection primer to introduce site-specific mutations into the plasmid. Both mutations were subsequently incorporated into the same strand by *in vitro* DNA synthesis. The selection primers eliminated the unique recognition sequence for the restriction enzyme *Apa*I in a nonessential region of the plasmid pGEX-2T, downstream and in-frame with the glutathione *S*-transferase coding sequence.

The mutagenized double-stranded DNAs were digested using *Apa*I restriction enzyme and introduced by transformation into *E. coli* DH5α. Mutations were confirmed by double-stranded DNA sequencing performed according to Sanger et al. (1977).

Expression of Fusion Proteins and Enzyme Purification. The expression of the recombinant wild-type and mutated enzymes was obtained after inducing the cultures with IPTG. The cultures were grown at 37 °C under shaking up to stationary phase, and the presence of the mutated proteins in the cell lysates was checked by SDS−PAGE according to Laemmli (1970). The acylphosphatase/glutathione *S*-transferase fusion proteins were purified as previously reported (Modesti et al., 1995) with minor modifications in order to obtain the reduced (−SH) form of the enzyme. The purified fusion proteins were checked by Western blot analysis. Thrombin cleavage was performed by incubating each fusion protein at room temperature for 1 h with 1:1000 (w/w) human thrombin in 50 mM tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$. Each cleaved acylphosphatase mutant was separated from both the glu-

tathione *S*-transferase and the corresponding uncleaved fusion protein by gel filtration as previously reported (Modesti et al., 1995). Fractions containing the wild-type or mutant acylphosphatases were pooled, concentrated by ultrafiltration, and analyzed for purity by SDS−PAGE.

Protein Determination and Amino Acid Analysis. Protein concentration was measured by either amino acid analysis or UV absorption using $A^{1\%}_{1cm,280} = 14.2$ for both native and mutated enzymes. Amino acid analyses were performed using a Carlo Erba Model 3A29 amino acid analyzer equipped with a Hewlett-Packard HP3395 computing integrator as previously described (Manao et al., 1985). Values for serine and threonine were corrected for degradation during sample hydrolysis.

Acylphosphatase Activity Measurements. Acylphosphatase activity was determined by a continuous optical test at 283 nm and 25 °C, using 5 mM benzoyl phosphate as a substrate dissolved in 0.1 M acetate buffer, pH 5.3, as previously reported (Ramponi et al., 1966). The activity of the Asn41 mutants was measured by adding to the test mixture an amount of enzyme over 1000-fold higher as compared to that used in the standard assay (0.010−0.015 mg/mL).

Equilibrium Dialysis Experiments. Equilibrium dialysis was performed as previously described (Taddei et al., 1994a) at pH 5.3 and an enzyme concentration of about 7−12 mg/ml, using 50 μL of both enzyme and dialysis solution; the latter contained varying concentrations of inorganic phosphate and [^{32}P]P_i at a specific radioactivity of 0.7 mCi/mmol. The samples were stored overnight at 5 °C; these conditions had previously been proven as largely suitable to attain equilibrium. After equilibrium was established, the ^{32}P radioactivity was measured in each dialysis compartment, and the data were processed by Scatchard analysis. Uncertainties were estimated by least-squares fitting and expressed as standard deviations.

^{1}H NMR Spectroscopy. The experiments were carried out at 600 MHz and 30 °C (or 70 °C, in the case of the thermally denatured enzyme) on an AMX600 Bruker NMR spectrometer. The wild-type and mutant enzyme samples, at a protein concentration of 5−10 mg/mL, were prepared in 50 mM acetate-d_3 buffer/D_2O, pH 3.8, and subsequently used for recording one-dimensional ^{1}H NMR spectra as previously reported (Taddei et al., 1995). 1,4-Dioxane was used as an internal shift reference at 3.74 ppm.

CD Experiments. CD experiments were performed on a Jasco Model. J720 spectropolarimeter equipped with a thermostated water bath, in the 205−250 nm absorption region using a 0.1 cm path-length cell. The samples were prepared in 50 mM acetate buffer, pH 3.8, at a protein concentration of 0.10−0.15 mg/mL. The actual temperatures inside the cell were monitored by a thermocouple during the thermal denaturation. In the urea denaturation experiments, protein samples were preincubated at 37 °C for 1 h in the presence of urea concentrations ranging between 0 and 6 M. CD spectra were base line corrected against solvent at different temperatures and urea concentrations. The data are reported as mean residue ellipticity, [θ]. The parameters describing thermal and urea denaturation transitions were determined as previously described (Pace, 1990; Taddei et al., 1994b). Uncertainties were estimated from least-squares fitting and expressed as standard deviations.

Table 1: Sequences of the Oligonucleotides Used for the Production of the Mutated Enzymes[a]

mutant	oligonucleotide sequence
Thr42Ala	5′ GTCAAGAAC**GCC**AGCAAAGG 3′
Thr46Ala	5′ CAGCAAAGGT**GCC**GTGACCGGC 3′
Asn41Ala	5′ GGGTCAAG**GCC**ACCAGCAAAGG 3′
Asn41Ser	5′ GGGTCAAG**AGC**ACCAGCAAAGG 3′
Asn41Gln	5′ GGGTCAAG**CAG**ACCAGCAAAGG 3′

[a] The mutated codons are in boldface type.

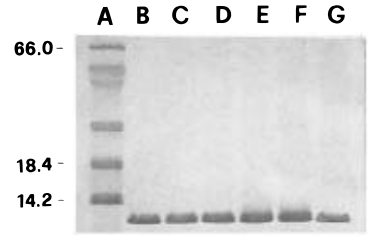

FIGURE 2: SDS−PAGE of the purified wild-type (lane B), Thr42Ala (lane C), Thr46Ala (lane D), Asn41Ala (lane E), Asn41Ser (lane F), and Asn41Gln (lane G) recombinant acylphosphatases. Lane A: standard molecular weight proteins.

RESULTS

Five oligonucleotides harboring the mutated codon for Thr and Asn, respectively (Table 1), were synthesized to perform an oligonucleotide-directed mutagenesis of acylphosphatase muscular isoenzyme by the USE method (Deng & Nickoloff, 1992); this was done by using the pGEX-AP construct as a template (see Materials and Methods). Five plasmids harboring the mutated genes were obtained, where Thr42 or Thr46 was replaced by alanine (Thr42Ala mutant and Thr46Ala mutant, respectively) and Asn41 was replaced by either alanine, glutamine, or serine (Asn41Ala mutant, Asn41Gln mutant, and Asn41Ser mutant, respectively). The sequences coding for the mutated acylphosphatases were separately amplified in the *E. coli* strain DH5α. Each mutated DNA was analyzed by sequencing according to Sanger's method and used to transform the expression *E. coli* strain TB1. Induction of expression was achieved by adding 1 mM IPTG to the growth medium. After induction, the bacterial cells were lysed by sonication and the clear lysates run in 15% SDS−PAGE. The Western blot analysis performed using polyclonal anti-recombinant muscle acylphosphatase antibodies (data not shown) demonstrated the absence, in the purified enzymes, of any immunoreactive material other than the enzyme itself. Six large bands were evident, corresponding to the five mutated proteins and to the wild-type recombinant acylphosphatase, respectively. A negative control prepared using strain TB1 transformed with pGEX-2T without the insert was also run. The supernatants from the sonicated cultures were purified by affinity chromatography and G75M gel filtration chromatography (see Materials and Methods). Figure 2 shows 15% SDS−PAGE of the purified recombinant enzymes. The purified wild-type and mutant enzymes were characterized for structural, kinetic, and stability properties.

Table 2 summarizes the main kinetic parameters calculated for all purified enzymes using benzoyl phosphate as a substrate and inorganic phosphate as a competitive inhibitor. The pH-optimum, apparent K_m, and K_i values elicited by the Thr42 and Thr46 mutants are remarkably similar to those of the wild-type enzyme, whereas the specific activity values

Table 2: Main Kinetic Parameters Calculated from Activity Measurements and Equilibrium Dialysis Experiments[a]

enzyme	specific activity (IU/mg)	pH-optimum	K_m (mM)	K_i (mM)	K_d (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
wild type	6500	4.8−5.8	0.36 ± 0.04	0.75 ± 0.07	0.47 ± 0.03	3400.0
Thr42Ala	4700	4.7−5.7	0.70 ± 0.06	1.17 ± 0.12	nd	1264.6
Thr46Ala	5000	4.8−5.8	0.66 ± 0.06	1.47 ± 0.07	nd	1426.7
Asn41Ala	10	4.9−5.9	0.33 ± 0.01	nd	0.14 ± 0.01	5.6
Asn41Ser	10	4.9−5.9	0.23 ± 0.01	nd	0.19 ± 0.02	8.0
Asn41Gln	3	4.9−5.9	0.55 ± 0.02	nd	1.51 ± 0.28	1.0

[a] One IU is defined as the catalytic activity which hydrolyzes 1 μmol/min of benzoyl phosphate, at pH 5.3 and 25 °C. The pH-optimum was calculated at 25 °C in 0.1 M acetate buffer, pH 3.7−6.5, and in 50 mM 3,3-dimethylglutarate buffer, pH 6.0−7.5. The other parameters were determined in 0.1 M acetate buffer, pH 5.3. K_m and K_i were calculated kinetically using benzoyl phosphate and inorganic phosphate as substrate and competitive inhibitor, respectively. K_d values were determined by equilibrium dialysis (see Materials and Methods). nd = not determined.

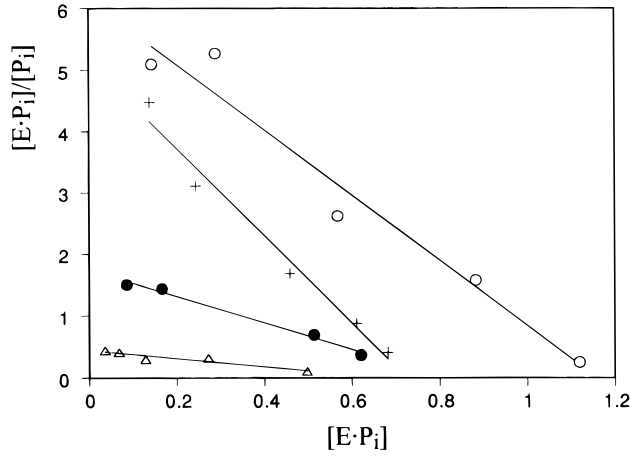

FIGURE 3: Scatchard plots determined from the equilibrium dialysis experiment in the presence of varying [^{32}P]P_i, in 50 mM acetate buffer, pH 5.3. (●) Wild-type acylphosphatase; (+) Asn41Ala mutant; (△) Asn41Gln mutant; (○), Asn41Ser mutant.

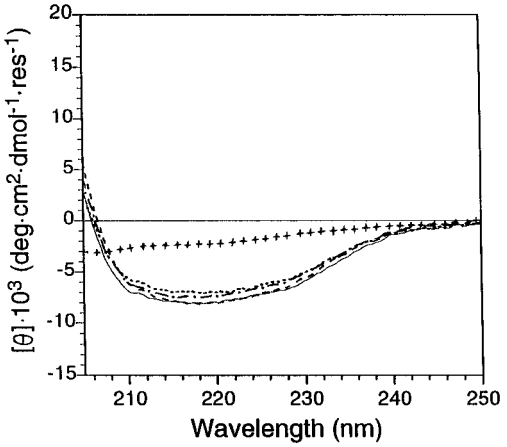

FIGURE 4: Comparison of the 205−250 nm regions of acylphosphatase CD spectra recorded in 50 mM acetate buffer, pH 3.8, at 30 °C and an enzyme concentration of 0.10−0.15 mg/mL. Wild type (—); Thr42Ala mutant (—·—); Thr46Ala mutant (···); Asn41Gln mutant (- - -). Thermally denatured (70 °C) wild-type enzyme is represented as (+ + +).

appear only partially reduced (about 72% and 77%, respectively, as compared to that of the wild-type enzyme). The three Asn41 mutants, though showing apparent K_m values very similar to those of the other enzymes, were almost totally inactive. For the Asn41Ala and Asn41Ser mutants, a specific activity of about 10 (0.15% as compared to the wild-type specific activity) was calculated by adding over a hundredfold higher amount of each enzyme mutant to the standard assay test. Similarly, under these conditions, the calculated activity of the Asn41Gln mutant was about 3, corresponding to nearly 0.05%, as compared to the wild-type specific activity. The strict similarity in substrate binding capability between the three Asn41 mutants and the wild-type enzyme was confirmed by performing an equilibrium dialysis experiment in the presence of radioactive inorganic phosphate, a well-known enzyme competitive inhibitor. The data were subjected to Scatchard analysis (Figure 3) and the apparent K_d values calculated from the slope of the $[E''P_i]/[P_i]$ versus $[E''P_i]$ straight lines fitted on the experimental points. The enzyme concentration in each experiment, as indicated by the intercept on the *x* axis, is in good agreement with that effectively used. The K_d values of all Asn41 mutants were similar to that of the wild-type enzyme and to the K_i values calculated kinetically for both the wild-type enzyme and the Thr mutants (Table 2).

Contrary to the Asn41 mutants, the high specific activity values of both Thr mutants suggest that their fold is not significantly different from that of the wild-type enzyme. To assess the absence of any major conformational change possibly responsible for the loss of activity, all mutant enzymes were subjected to structural investigation. The 600 MHz ^{1}H NMR one-dimensional spectra of the wild-type and mutant enzymes show a notable chemical shift dispersion, indicating the presence of persistent, well-established tertiary structures (data not shown). Furthermore, considerable protection of backbone amide protons (resonances in the 7.5−9.5 ppm region) against exchange with the deuterated solvent, characteristic of folded structures (Wüthrich, 1986), is observed. In the spectrum of the thermally denatured wild-type enzyme, at 70 °C (Modesti et al., 1995), the chemical shift dispersion was completely lost, and the amide proton resonances fully disappeared. The ^{1}H NMR spectra of the folded enzymes are noteworthy similar to each other, even in the case of the almost completely inactive Asn41 mutants (data not shown). This result was confirmed by the far-UV CD spectra shown in Figure 4. The differences between the spectra of Thr42Ala, Thr46Ala, Asn41Gln mutants and wild-type are not significant, indicating the presence of a comparable, well-defined, "native-like" secondary structure in all enzymes. The far-UV CD spectrum of the thermally denatured (70 °C) wild-type acylphosphatase is also reported in Figure 4: the fall of CD signal in the 205−225 nm absorption region indicates a loss of stable secondary structure. The far-UV CD spectra of the Asn41Ala and Asn41Ser mutants, though not shown in Figure 4, looked very similar to the wild-type one.

The thermal and urea stabilities of the wild-type and mutated enzymes were studied by following the CD signal at 222 nm. The ellipticity measured at this wavelength

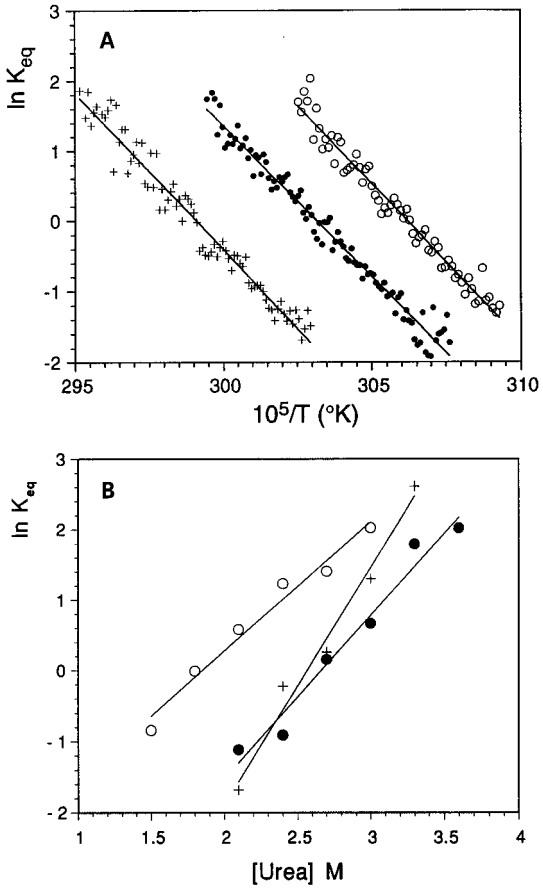

FIGURE 5: Natural logarithms of the equilibrium denaturation constants calculated from CD data plotted as a function of (A) the reciprocal of temperature and (B) the molar urea concentration. Panel A: (●) wild type; (○) Thr46Ala; (+) Asn41Ala. Panel B: (●), wild type; (○) Thr42Ala; (+) Asn41Ser.

reflects the changes of secondary structure, particularly the α-helical content. Changes in ellipticity can be monitored under increasing denaturing conditions, allowing one to follow the denaturation transition. All enzymes studied elicited a decrease of signal absolute intensity at 222 nm as part of a two-state denaturation transition, either in the presence of urea or under increasing temperature (data not shown). Denaturation equilibrium constants were calculated from the CD signal intensities at each temperature or urea concentration as previously reported (Taddei et al., 1994b). Figure 5 shows the van't Hoff isochore (A) and isotherm (B) plots, as resulted from fitting the data obtained in thermal and urea denaturation experiments, respectively. All mutants were subjected to this analysis, however, for an easier graphical representation, only the data relative to Asn41Ala/Ser, Thr42Ala/Thr46Ala, and wild-type enzyme are shown in Figure 5. The thermodynamic parameters relative to the unfolding transition, as calculated from thermal and urea denaturation CD experiments, are reported in Table 3. The c_m and t_m values (urea concentration and temperature, respectively, required to unfold 50% of enzyme molecules) found at pH 3.8 for the Asn41 mutants appear substantially identical to those calculated for the wild-type enzyme as well as to previously reported data (Taddei, et al., 1994b). The c_m and t_m values found for both Thr mutants were significantly reduced. Protein stability, as indicated by the $\Delta G(H_2O)$ value, appears very similar, within experimental error, for all enzymes except for the Thr42Ala mutant; this

Table 3: Thermodynamic Parameters of Thermal and Urea Denaturation Transitions Derived from Cd Data[a]

enzyme	c_m (M)	ΔG (H_2O) (kJ mol^{-1})	t_m (°C)	ΔH (kJ mol^{-1})
wild-type	2.83 ± 0.21	16.9 ± 1.5	56.8 ± 1.6	361.6 ± 16.8
Thr42Ala	1.85 ± 0.17	8.8 ± 0.4	52.5 ± 1.9	371.8 ± 17.1
Thr46Ala	1.91 ± 0.36	14.2 ± 2.8	53.6 ± 1.8	373.0 ± 18.8
Asn41Ala	2.93 ± 0.12	17.8 ± 0.6	61.3 ± 2.2	371.3 ± 18.7
Asn41Ser	2.56 ± 0.21	22.3 ± 1.9	59.0 ± 2.0	375.0 ± 20.5
Asn41Gln	2.88 ± 0.31	20.9 ± 2.9	60.2 ± 2.1	406.1 ± 20.1

[a] Experimental Conditions Are Described in the text. c_m and t_m are the urea concentration and temperature, respectively, required to denature 50% of the enzyme molecules. $\Delta G(H_2O)$ is the unfolding Gibbs free energy in the absence of urea, extrapolated by $\Delta G = \Delta G(H_2O) - m$[urea], where m is empirically related to the apolar surface area made accessible upon unfolding (Pace, 1990).

mutant displays a $\Delta G(H_2O)$ value which is about half of that shown by all other enzymes.

Reversibility of thermal and urea unfolding (over 90%) was demonstrated for all enzymes, measuring a native-like CD signal at 222 nm after cooling or diluting unfolded samples. A similar behavior was also observed by ^{1}H NMR spectroscopy: the spectra of the thermally unfolded proteins (70 °C), lacking any chemical shift dispersion, completely recovered their "native-like" features after cooling (data not shown).

DISCUSSION

Asn41, Thr42, and Thr46 are invariant residues in all acylphosphatase isoenzymes so far sequenced. From the three-dimensional solution structure of horse muscle enzyme (Pastore et al., 1992), it turns out that these residues are located in strict proximity to Arg23, the proposed main phosphate binding site of acylphosphatase (Taddei et al., 1994a), as can be seen in Figure 1. In particular, the ^{1}H NMR structure data indicate that Asn41 interacts, by hydrogen bonding, with Tyr98 and Arg97, the two C-terminal residues whose involvement in stabilizing the active site conformation has recently been reported (Stefani et al., 1991; Taddei et al., 1995). A previous study (Satchell et al., 1972) suggested the presence, at the acylphosphatase active site, of a neutral group possibly involved in binding and orienting the water molecule responsible for nucleophilic attack to the carboxyl phosphate bond during substrate hydrolysis. Available structural data suggest that either Thr42, Thr46, or Asn41, strictly conserved in all acylphosphatases so far sequenced, could be candidates for such a role. Consequently, we prepared enzyme mutants involving these residues, studying their kinetic, structural, and stability properties.

Contrary to Thr42 and Thr46, whose specific activities are not considerably different from that of the wild-type enzyme, the three Asn41 mutants, particularly Asn41Gln, elicit dramatically reduced specific activity values. The loss of activity cannot be ascribed to any global change in the three-dimensional structure of each mutant; this is demonstrated both by the considerable chemical shift dispersion and the persistent amide proton resonances in the ^{1}H NMR spectra of the mutants and by the presence of CD signals characteristic of folded structures. The ^{1}H NMR and CD spectra of the thermally denatured wild-type enzyme are substantially different from those acquired at 30 °C (data

not shown and Figure 4), further confirming the absence of major conformational changes in all mutants. Moreover, the ^{1}H NMR and CD spectra of all mutants are reasonably similar to those of the wild-type enzyme, suggesting that native-like overall folds do exist. In spite of the above reported structural data, the presence of local perturbations at the active site level able to abolish the enzyme catalytic efficiency cannot be completely excluded. The values of both the K_d for inorganic phosphate (a well-known enzyme competitive inhibitor) and the apparent K_m for benzoyl phosphate (a synthetic substrate), as calculated by equilibrium dialysis and kinetic analysis, respectively, make unlikely the presence of significant local structural changes at the active site. In fact, the inactive Asn41Ala, Asn41Ser, and Asn41Gln mutants show apparent K_m and K_i values comparable to those of both the wild-type acylphosphatase and the two Thr mutants. These data, together with the ^{1}H NMR and CD findings, strongly support the existence, in the Asn41 mutants, of a native-like structure even at the active site level. The k_{cat}/K_m values, shown in Table 2, further underline that Asn41 substitution strongly impairs acylphosphatase catalytic efficiency. The dramatic loss of activity of Asn41 mutants, whose ability to bind substrate appears unchanged, can therefore be specifically ascribed to the absence of the Asn41 side chain. The above reported results highlight the extreme susceptibility of the active site geometry even to a particularly conservative substitution, such as Asn to Gln. Indeed, full comprehension of the Asn41 mechanistic role in enzyme catalysis as the residue involved in binding and orienting the water molecule responsible for substrate hydrolysis requires further investigation. In fact, the possibility that Asn41 interacts through hydrogen bonding with the substrate phosphate moiety cannot be excluded; this interaction might correctly orient the substrate in the enzyme active site. To definitely assess the Asn41 role in enzyme catalysis, a high-resolution structure determination of acylphosphatase in the presence of the substrate (or competitive inhibitor) appears necessary.

Thermal and urea stabilities of Thr and Asn mutants were also investigated by CD experiments. These experiments were primarily performed to assess the stability and behavior of the Asn41 mutants under increasing denaturing conditions. As it can be seen from Table 3, the thermodynamic parameters relative to the unfolding transition of the Asn41 mutants, as compared to those of the wild-type enzyme, lead to the conclusion that the introduced mutations have no significant effect on enzyme stability, further confirming that the inactivation of those mutants is merely due to the absence of a catalytically important side chain. Surprisingly, the Thr mutants (particularly the Thr42Ala mutant) elicited a sharply reduced stability together with a higher sensitivity to temperature and urea, as expressed by the $\Delta G(H_2O)$, t_m, and c_m values, respectively. The decreased stability of the Thr mutants can be explained in light of the acylphosphatase structural data presently available (Pastore et al., 1992). Thr42 is the C-terminal residue of the second β-strand, and Thr46 is part of the 43−46 loop connecting the second and third β-strands. The five best acylphosphatase structures derived from ^{1}H NMR data indicate that both threonine residues are exposed to the solvent. Taking into account the uncertainty of these structures, Thr42 forms hydrogen bonds with Thr46 and Lys44 or Gly45, at both the side-chain and the backbone level; these interactions are likely to form a network possibly involved in loop stabilization. It is well-known that substitutions of solvent-exposed residues usually have little or no effect on protein stability, unless the replaced side chain plays an important, specific role, *e.g.*, forming salt bridges or stable hydrogen bonds (Creighton, 1993). Since it has been shown that the Thr42Ala mutant elicits kinetic properties very similar to those of the wild-type enzyme, it might be supposed that the replacement of such a hydrophilic side chain with a hydrophobic one affects muscle acylphosphatase stability *via* destabilization of the 43−46 loop.

The reported results underline (i) the central role of Asn41 (an invariant residue in all acylphosphatases sequenced so far) in the enzyme catalytic mechanism, (ii) the persistence, in the inactive Asn41 mutants, of substrate binding ability, (iii) the substantially unchanged structural stability of the Asn mutants, and (iv) the possible role of Thr42 and, to a less extent, Thr46 as residues stabilizing the overall three-dimensional structure of the enzyme. In light of these results, Asn41 mutants, in particular the Asn41Gln mutant, can be considered excellent candidates as negative dominants for use in cell biology experiments aimed to better understand the physiological actions of acylphosphatase.

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