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The organ common-type (CT) isoenzyme of acylphosphatase is inactivated by Woodward's reagent K (WRK) (*N*-ethyl-5-phenylisoxazolium-3′-sulphonate) at pH 6.0. The inactivation reaction follows apparent pseudo first-order kinetics. The dependence of the reciprocal of the pseudo first-order kinetic constant (k_{obs}) on the reciprocal WRK concentration reveals saturation kinetics, suggesting that the WRK forms a reversible complex with the enzyme before causing inactivation. Competitive inhibitors, such as inorganic phosphate and ATP, protect the enzyme from WRK inactivation, suggesting that this reagent acts at or near to the enzyme active site. The reagent–enzyme adduct, which elicits a strong absorption band with λ_{max} at 346 nm, was separated from unreacted enzyme by reverse phase HPLC and the modified protein was cleaved with endoproteinase Glu-C to produce fragments. The HPLC fractionation gave two reagent-labelled peptides (peak 1 and peak 2) that were analysed by ion-spray MS and sequenced. The former is VFFRKHTQAE (residues 20–29 of human CT acylphosphatase) and the latter IFGKVQGVFFRKHTQAE (residues 13–29). MS demonstrated that both peptides are WRK adducts. A fragment ion with m/z of 1171, which is present in the mass spectrum of peak 1, has been identified as a WRK adduct of the peptide fragment 20–26. The λ_{max} at 346 nm of WRK adduct suggests that the modified residue is His-25. Five recombinant enzymes mutated in residues included in the 20–29 polypeptide stretch have been produced. Analysis of their reactivities with WRK demonstrates that His-25 is the molecular target of the reagent as its modification causes the inactivation of the enzyme. Since both His-25 → Gln and His-25 → Phe mutants maintain high catalytic activity, we suggest that the observed enzyme inactivation is caused by the reagent (covalently bound to His-25), which shields the active site.

INTRODUCTION

Acylphosphatase (E.C. 3.6.1.7) is an enzyme with a low M_r (98 amino acid residues), which catalyses the hydrolysis of compounds containing the carboxyphosphate bond. The enzyme acts both on low molecular-mass compounds (metabolites such as 1,3-bisphosphoglycerate and carbamoyl phosphate and synthetic acyl phosphates [1]), and on proteins containing phosphorylated carboxy groups, such as Na^+/K^+-ATPase [2] and Ca^{2+}-ATPase [3], which mediate membrane ion transport. Recently, this type of protein phosphorylation (aspartyl phosphate) has been discovered in several bacterial species as well as in yeast, where one protein of the two-component system (a system involved in adaptation to changes in cell environment) is phosphorylated on an aspartate residue [4,5]. Two isoenzymes of acylphosphatase have been discovered and their amino acid sequences have been determined [6,7]. These two isoenzymes, named the organ common-type (CT) and muscle-type (MT), originate from two distinct genes and elicit about 50 % sequence similarity. Both are expressed in animals in a tissue-specific manner [8,9]. Recently, CT-isoenzyme crystals have been produced [10] and X-ray diffraction analysis has revealed that the main fold of CT acylphosphatase is very similar to that of the MT isoenzyme [11].

The physiological role of acylphosphatase is not yet completely understood. However, several reports have demonstrated that the enzyme is involved in the control of the ionic conditions in the cell [12,13], probably acting on the aspartyl phosphate formed during the functioning of membrane-ion pumps [14,15]. Other reports have indicated that the enzyme is involved in cell differentiation [16–18]. In particular, a very recent report on the differentiation of the K562 cell line, when stimulated by PMA (which induces megakaryocytic differentiation), or by aphidicolin or hemin (which stimulate erythrocytic differentiation), has demonstrated that while the MT isoform showed an average 10-fold increase independently of the differentiating agent used, only hemin treatment caused a similar increase in the CT isoform, suggesting a different role for the two isoenzymes in the cell [18]. Finally, the demonstration that the tertiary structure of the enzyme is identical with that of the RNA-binding domain of nuclear RNA-binding proteins [19,20] and that the enzyme possesses nucleolytic activity [21], together with the observation that the CT isoenzyme is able to migrate into the nucleus during apoptosis [22], all suggest that the enzyme is involved in critical biological functions.

This paper deals with the inactivation of CT acylphosphatase caused by Woodward's reagent K (WRK) (*N*-ethyl-5-phenylisoxazolium-3′-sulphonate). This reagent has been generally used as a chemical modifier of enzyme carboxy groups. The structure of WRK incorporates some features common to good acylphosphatase substrates, such as benzoyl phosphate, since it contains an aromatic benzene ring and an anionic moiety and, therefore, could be expected to have some affinity for the acylphosphatase active site.

MATERIALS AND METHODS

Human CT-recombinant acylphosphatase isoenzyme was prepared as described previously [23]. WRK and endoproteinase Glu-C were purchased from Sigma. The pGEX-KT vector, oligonucleotides and the unique restriction-site-elimination mutagenesis kit were obtained from Pharmacia. Sequenase was obtained from the U.S. Biochemical Co. [α-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont-NEN. All other reagents were the purest commercially available.

Abbreviations used: WRK, Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulphonate); CT, common-type; MT, muscle-type; WT, wild-type; TFA, trifluoroacetic acid.

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Protein determination

Protein concentration was assayed using the bicinchoninic acid method (Sigma).

Enzyme assay and kinetic parameters

Acylphosphatase activity was assayed using a continuous optical recording with benzoyl phosphate as substrate [24]. The substrate was dissolved in 0.1 M sodium acetate buffer, pH 5.3, and the incubation was performed at 25 °C. The main kinetic parameters (K_m and V_{max}) were determined by measuring the initial rates at different substrate concentrations (ranging from 0.1–5 mM). The experimental data were analysed using the Michaelis equation and a non-linear fitting program (FigureP; Biosoft). Enzyme inhibition by P_i (a well-known competitive inhibitor of the enzyme) was determined measuring initial hydrolysis rates at differing substrate (range 0.1–5 mM) and P_i (range 0.5–4 mM) concentrations. The apparent K_m values measured at the various P_i concentrations were plotted against P_i concentration to calculate the K_i value. All initial rate measurements were carried out in triplicate at least.

Reaction of CT acylphosphatase with WRK

Freshly prepared stock solutions of WRK in 1 mM HCl were used. The modification reactions were performed at 25 °C in 0.15 M sodium cacodylate buffer, pH 6.0. The incubation mixture (180 μl) contained 85 μM enzyme and WRK ranging from 0.1–2.4 mM (final concentration). At different time intervals, the reaction was quenched by the addition of 10 μl of 2.5 M sodium acetate and the mixtures were assayed for enzyme activity. In experiments performed with some mutated enzymes, the final concentration of WRK was increased in order to measure inactivation rates. Ligands (P_i or ATP) were added before the addition of the WRK stock solutions. The number of modified residues per enzyme molecule was determined as follows. The enzyme solutions were incubated in separate vials with 0–20 mM WRK (final concentration) at 25 °C. After 2 min, the reaction was stopped by the addition of sodium acetate (0.13 M, final concentration). The samples (after 10 min) were applied to a Sephadex G25 column (1 cm × 30 cm) equilibrated with 0.05 M sodium cacodylate buffer, pH 6.0, containing 0.6 M NaCl. The column was eluted at a flow rate of 40 ml/h, and 2 ml fractions were collected. The void volume fractions were pooled and the absorption spectrum was recorded. All enzyme samples treated with WRK had an absorption band with a λ_{max} at 346 nm, which was not present in the untreated samples. The levels of protein modified by WRK were estimated by the increase in absorbance at 340 nm (ϵ 7000 $M^{-1} \cdot cm^{-1}$; [25]).

Endoproteinase Glu-C digestion and purification of peptides

Endoproteinase Glu-C digestion was performed as follows. The enzyme [wild-type (WT) or modified, 700 μg] was dissolved in 0.1 M sodium phosphate buffer (pH 7.8)/1 M guanidinium chloride, endoproteinase Glu-C (34 μg) was added and the mixture was incubated at 30 °C for 16 h. The sample was dried in a vacuum evaporator (Savant), dissolved in 20 μl of water and applied on to a Vydac Protein and Peptide C_{18} HPLC column (4.6 mm × 250 mm, 5 μm). Peptides were eluted using a trifluoroacetic acid (TFA)/acetonitrile gradient, as described in the legend to Figure 4. The A_{214} and A_{346} were recorded and fractions were collected manually.

Sequence analysis and MS

The amino-acid sequences were determined using the Milligen Mod 6600 Protein Sequencer. Peptides were covalently immobilized on poly(vinylidene difluoride)–arylamine membranes using a carbodiimide-based attachment method, and were then applied to the sequencer reactor.

Ion-spray mass spectra of WRK-labelled peptides in positive ion mode were acquired on a Perkin-Elmer Sciex API III mass spectrometer (Sciex Co., Thornhill, Ontario, Canada) using an ion-spray voltage of 5.5 kV, an orifice voltage of 60 V and a scan range of 500–1600 m/z.

Site-directed mutagenesis of acidic residues

Site-directed mutagenesis was performed using a unique-restriction-site elimination kit according to the manufacturer's instructions, with minor modifications to the pGEXKT-CT acylphosphatase construct. CT acylphosphatase cDNA was inserted into the pGEXKT expression vector as described previously [23], and this was used as a template for the unique-restriction-site elimination oligonucleotide-directed mutagenesis reaction [26]. The following amino acid residues were substituted: Arg-23 → His, Lys-24 → Gln, His-25 → Gln, His-25 → Phe and Glu-29 → Gln using five different 25-base-long synthetic oligonucleotides to introduce single mutations, together with the *Apa*I restriction-site-eliminating oligonucleotide. The mutated genes were completely sequenced using the method of Sanger [26a] to verify that a single mutation had occurred. The expressed fusion proteins were purified by single-step affinity chromatography using a glutathione-linked agarose resin as described previously [23]. The presence of a thrombin-cleavage site at the joining point of the chimaera protein permitted the cleavage of the recombinant enzyme from the fusion protein using purified bovine thrombin. Thrombin cleavage was performed by incubating each fusion protein on ice for 30 min with 1:500 (w/w) thrombin in 50 mM Tris/HCl buffer (pH 8.0)/150 mM NaCl/0.1 % (w/v) 2-mercaptoethanol/2.5 mM $CaCl_2$. Each cleaved CT acylphosphatase mutant was separated from uncleaved fusion protein by affinity-chromatography on glutathione-linked resin.

RESULTS

CT acylphosphatase inactivation by WRK

Incubation of CT acylphosphatase with WRK at pH 6.0 and 25 °C resulted in a time- and concentration-dependent loss of enzymic activity (Figure 1A). Since some authors have observed the formation of reversible WRK–enzyme complexes before covalent-bond formation [27,28], we applied the following model, proposed by Petra [27], in order to analyse the mechanism of acylphosphatase inactivation by WRK:

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \overset{k_2}{\rightarrow} E - I$$

where, E is free enzyme; I is WRK; E·I is the enzyme–WRK reversible complex; E–I is the enzyme–WRK covalent complex; K_I is the apparent E·I dissociation constant; k_2 is the intrinsic rate constant for covalent modification of CT acylphosphatase.

Using the method of Petra [27], we derived the following equation:

$$\ln \frac{[E']}{[E_0]} = -k_{obs} \times t$$

where $[E'] = [E] + [E \cdot I]$, $[E_0]$ is the total enzyme concentration, and $k_{obs} = k_2/(K_I/[I] + 1)$. The inactivation rate can be obtained

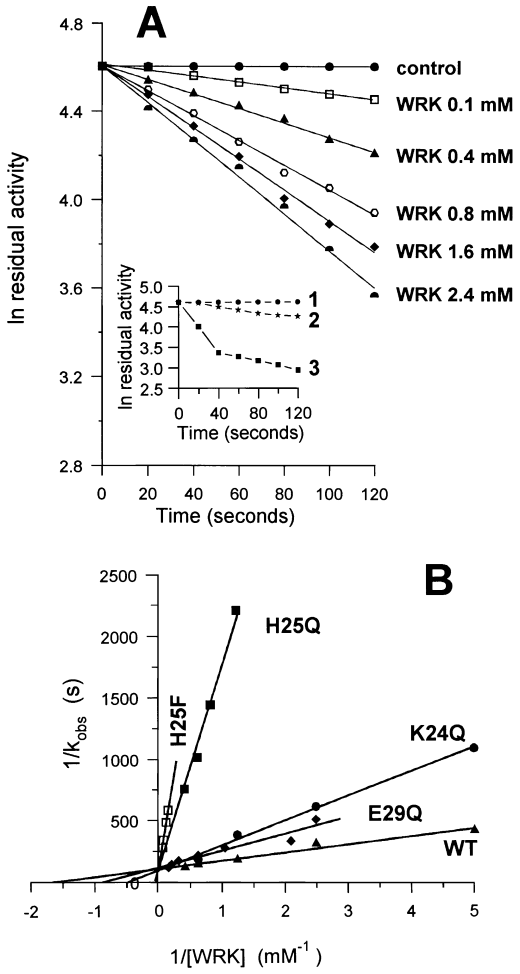

Figure 1 Inactivation of CT acylphosphatase by WRK

(**A**) WT CT acylphosphatase (85 μM, 180 μl final volume) was treated with 0.1, 0.4, 0.8, 1.6 or 2.4 mM WRK (final concentrations) at 25 °C in 0.15 M sodium cacodylate buffer, pH 6.0. At various time intervals, aliquots were removed and assayed for residual activity. The inset shows the effect of the competitive inhibitor P_i on the inactivation of the WT CT acylphosphatase. The experiments were performed with 85 μM enzyme in 0.15 M sodium cacodylate buffer, pH 6.0, in a final volume of 180 μl at 25 °C. Line 1, control; line 2, 5 mM WRK and 100 mM P_i; line 3, 5 mM WRK. (**B**) [WRK] dependence of the pseudo first-order rate constant (k_{obs}) for the WT and mutant enzymes. H25F, His-25 → Phe; H25Q, His-25 → Gln; K24Q, Lys-24 → Gln; E29Q, Glu-29 → Gln.

by plotting the ln percentage of residual activity versus time (t). Figure 1(A) shows that, at WRK concentrations in the range 0.1–2.4 mM, the inactivation reaction obeys pseudo first-order kinetics. At higher WRK concentrations, biphasic inactivation curves were observed (see the 5 mM WRK curve, Figure 1(A) inset). The apparent first-order kinetic constant (k_{obs}) values relative to the different WRK concentrations were calculated from all curves shown in Figure 1(A). The data were further analysed with the following equation [27]:

$$\frac{1}{k_{obs}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2}$$

Replotting $1/k_{obs}$, measured at various WRK concentrations (range 0.2–2.4 mM), against the reciprocal of WRK concentration, we observed saturation kinetics (the straight line fails to pass through the origin) (Figure 1B). This finding demonstrates

Table 1 Some kinetic parameters of recombinant WT and mutated human CT acylphosphatases

	V_{max} (μmol/min//mg)	K_m (mM)	K_i (P_i) (mM)
WT	4870 ± 89	0.14 ± 0.01	0.58 ± 0.03*
Arg23 → His	3.6 ± 0.1	n.d.	n.d.
Lys24 → Gln	2753 ± 22	0.45 ± 0.01	1.75 ± 0.07*
His25 → Gln	2785 ± 17	0.49 ± 0.03	1.96 ± 0.05*
His25 → Phe	6646 ± 73	0.25 ± 0.01	1.34 ± 0.03*
Glu29 → Gln	5249 ± 65	0.13 ± 0.01	0.53 ± 0.07*

* Competitive inhibition; n.d., not determined.

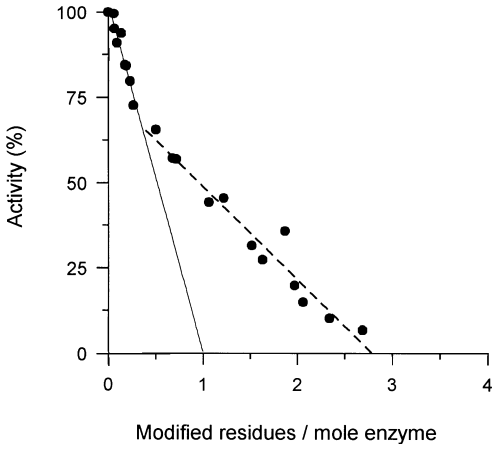

Figure 2 Stoichiometry of the modification of CT acylphosphatase by WRK

Residual activity was plotted against mol of modified residues/mol of enzyme. The enzyme (85 μM) was treated with 0–20 mM WRK in 0.15 M sodium cacodylate buffer, pH 6.0, in a final volume of 180 μl at 25 °C. After two min, the reaction was terminated and the protein was separated from the reagent by Sephadex G25 chromatography. The continuous line shows the initial enzyme inactivation caused by WRK with a single highly reactive group, with extrapolation to zero activity. The broken line shows the inactivation caused by the reaction of WRK with additional groups, with extrapolation to zero activity.

the formation of an enzyme–WRK reversible complex before the modification of the covalent enzyme, which brings about inactivation of the enzyme. Using the proposed model, we can calculate the apparent dissociation constant and the apparent first-order rate constant at saturation for the WT enzyme at 25 °C and pH 6.0 ($K_I = 0.59$ mM and $k_2 = 0.0094$ s^{-1} respectively).

The influence of P_i, a product of substrate hydrolysis which competitively inhibits the enzyme ($K_I = 0.58$ mM), and ATP ($K_I = 63$ μM, competitive inhibition) was studied. The inset of Figure 1(A) shows that P_i protects the enzyme from WRK-inactivation. ATP, which is a competitive inhibitor, also protects the enzyme from WRK inactivation (results not shown). The K_I of 0.59 mM found for WRK indicates that the affinity of the enzyme for WRK is very similar to that for P_i ($K_I = 0.58$ mM). This is probably due to the sulphonate moiety of WRK, which mimics the phosphate moiety of the substrate, thus interacting with the anion binding site of the enzyme.

Stoichiometry of enzyme modification

Figure 2 shows the stoichiometry of WRK incorporation into acylphosphatase as function of residual activity. It can be seen

that the plot is biphasic. In the former phase (0–30 % inactivation, 0–0.4 mM WRK), the experimental data fit to a straight line that intersects the abscissa at a point corresponding to the incorporation of 1 mol of WRK/mol of enzyme indicating that a single enzyme residue is involved in the inactivation. In the latter phase (inactivation values above 30 % produced by 0.5–20 mM WRK), the experimental data fit a straight line having a different slope. This line intersects the abscissa at a point corresponding to the incorporation of 3 mol of WRK/mol of enzyme, suggesting that, during this phase, two additional residues with lower reactivity are modified by WRK. Similar results have been observed for different enzymes in their reactions with WRK [27–31]. We noted that the competitive inhibitor P_i protected the enzyme from WRK inactivation at levels of 20 % residual activity (Figure 1A, inset), suggesting that all three reactive residues are protected.

Separation of the WRK–enzyme complex from unreacted enzyme

The acylphosphatase–WRK adduct was separated from unreacted enzyme by gel filtration on Sephadex G25 and reverse phase HPLC. Experimental conditions for enzyme modification and for adduct purification are described in the legend to Figure 3. Two peaks (peak-I and peak-II), were eluted at about 36 % acetonitrile concentration (Figure 3A). The fractions containing these two peaks were collected, dried, redissolved in water and their UV spectra were recorded. Peak I elicited an absorption band at 280 nm, due mainly to the presence of protein tyrosine and tryptophan residues (Figure 3B), and peak II produced an additional absorption band with λ_{max} at 346 nm (Figure 3C). Similar absorbance in the 340–350 nm range has been reported for several protein–WRK adducts [27,29–38]. The stoichiometry of WRK incorporation, measured using the method of [25], was 1.2 mol of WRK/mol of enzyme. Thus peak I is unreacted enzyme and peak II is WRK-derivatized enzyme.

Separation of the endoproteinase Glu-C fragments, sequence and MS analyses of WRK-labelled peptides

The upper two traces (A, B) of Figure 4 show the elution profile of endoproteinase Glu-C peptides obtained from the native enzyme after HPLC. The lower two traces (C, D) show the profile of the peptides obtained from the WRK-derivatized enzyme. The patterns differ mainly in the presence of two peaks (peak-1 and peak-2) seen in panel D, both with absorbancies at 214 and 346 nm (the latter is the λ_{max} of the protein–WRK adducts). The height of peak 1 is about twice that of peak 2. Amino-acid-sequence analysis of peak 1 gave: VFFRKHTQAE, while that of peak 2 gave: IFGKVQGVFFRKHTQAE. Peak 1 contained the sequence of residues 20–29, and peak 2 contained the sequence of residues 13–29 of human CT acylphosphatase.

Figures 5(A) and 5(B) show the ion-spray mass spectra of peak 1 and peak 2 respectively. The presence of the molecular ion with a mass of 1515.4 m/z, shown in Figure 5(A), confirms that a single site in peptide residues 20–29 was modified by WRK (the calculated molecular mass of the adduct is 1515.7). The mass spectrum of peak 2 (Figure 5B) shows a major peak having a mass of 1123.8 m/z. Since the calculated molecular mass of this peptide adduct is 2245.6, the 1123.8 m/z peak represents the double-charged $(M^+ + 2H)^{2+}$ ion of the WRK adduct with residues 13–29 of the acylphosphatase peptide. The molecular-ion peak (Figure 5B, inset) was not observed, probably because its yield was very low. Although mass and absorption spectra showed that both peptides were WRK adducts, gaps or unusual phenylthiohydantoin derivatives were not observed in the Edman sequencing. This is probably due to the instability of the WRK adduct in the strong acidic environment of the solid-phase Edman chemistry.

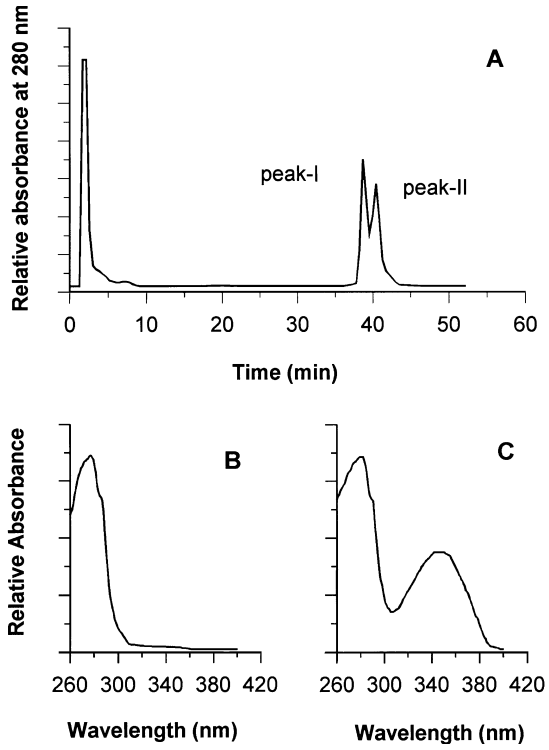

Figure 3 Separation of WRK-modified from non-modified CT acylphosphatase by HPLC

(**A**) The enzyme (5 mg) was treated with 0.2 mM WRK in 0.15 M sodium cacodylate buffer, pH 6.0, at 25 °C in a final volume of 3.6 ml. When the residual activity had decreased to approx. 70 %, the reaction was stopped by the addition of 150 μl of 2.5 M sodium acetate and the enzyme was separated from the reagents by gel filtration on a Sephadex G25 column (2.5 × 30 cm) equilibrated with 0.05 M sodium cacodylate (pH 6.0)/0.6 M NaCl, and eluted at a flow rate of 120 ml/h; 2.5 ml fractions were collected. The protein contained in the void volume was applied to a reverse-phase HPLC column (4.6 × 75 mm, 5 μm; Altex ULTRAPORE RPSC) equilibrated with 10 mM TFA in water. After washing with this solvent for 10 min at a flow rate of 0.8 ml/min, a 10 mM TFA in acetonitrile (solvent A) gradient was applied (0–10 min, 0 % solvent A, followed by 0–70 % solvent A over 60 min). (**B**) UV absorption spectrum of peak-I (non-modified enzyme) isolated in (**A**). (**C**) UV absorption spectrum of peak-II (WRK-modified enzyme) isolated in (**A**).

Both peptides contained a single acidic residue, i.e. Glu-29. As several published papers have indicated acidic residues as targets of WRK in proteins [27,29,30,34–36,38], we have considered the possibility that this residue could be the target of WRK in the inactivation of CT acylphosphatase. We observed that endoproteinase Glu-C easily produced the cleavage of the Glu-29–Gly-30 peptide bond, indicating that this acidic residue contains its γ-carboxy group in a free form, thus excluding the possibility that this residue is the target of WRK in CT acylphosphatase inactivation. In order to identify the actual WRK target residue in acylphosphatase, we considered the report of Llamas et al. [39]. This very valuable research showed that, in aqueous solutions, WRK rapidly produces a keto ketenimine which reacts easily with several chemical groups of protein side-chains. They observed that the reaction rates of WRK with model compounds containing imidazole, thiol, amino, phenoxide, carboxylate and carboxy groups are all pH-dependent. Taking the results obtained by these authors into consideration, we would expect imidazole to be more reactive than carboxyl and primary amino

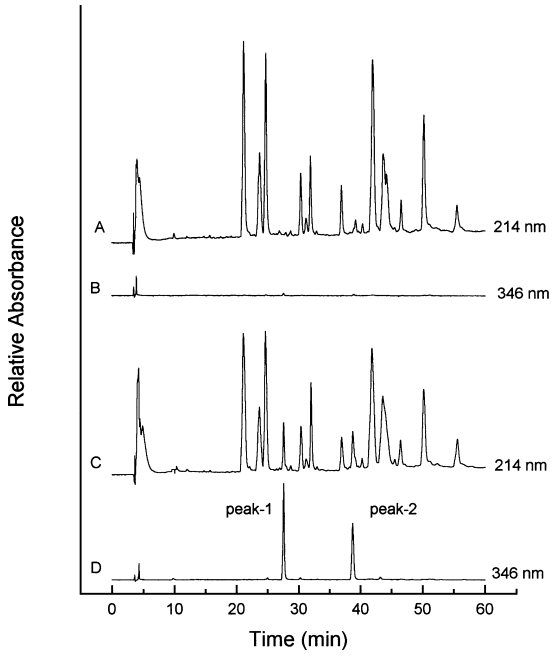

Figure 4 HPLC resolution of endoproteinase Glu-C digests

The two upper traces (**A**, **B**) are the elution profile of the unmodified enzyme. The two lower traces (**C**, **D**) are those of the WRK-modified enzyme (the modified enzyme contained about 1 mol of WRK/mol of enzyme). Both peak-I and peak-II enzymes (700 μg of each, prepared as described in the legend to Figure 3) were digested with endoproteinase Glu-C as described in the Materials and methods section. The digests were applied on to a reverse-phase HPLC column (4.6 × 250 mm, 5 μm; Peptide and Protein C_{18}, Vydac) equilibrated with 10 mM TFA in water at a flow rate of 0.72 ml/min. A 10mM TFA/acetonitrile gradient (0–27%) was applied over 60 min. The elution of WRK adducts was monitored at A_{346}. The WRK-labelled peptides (peak-1 and peak-2) were collected and dried under vacuum.

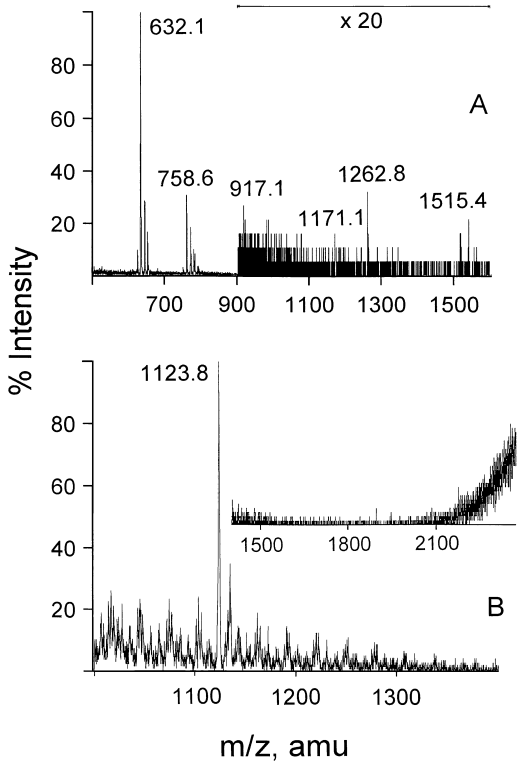

Figure 5 Mass spectra of WRK-labelled endoproteinase Glu-C peptides

Ion-spray mass spectra of (**A**) peak 1 and (**B**) peak 2.

groups, since we performed the acylphosphatase–WRK inactivation experiments at pH 6.0 [39]. In addition, the λ_{max} at 346 nm of the band present in the spectrum of the enzyme–WRK adduct (Figure 3C) is very close to that observed by these authors for the imidazole–WRK adducts (342 nm for conjugated acid and 348.5 nm for conjugated base, $pK'_a = 4.5$). The enol ester produced in the reaction of WRK with a carboxy group should have a λ_{max} close to 267 nm, and the primary amine–WRK adduct should have a λ_{max} at 246.5 nm (conjugated acid) and 319 nm (conjugated base, the pK'_a of the methylamine–WRK adduct is 6.81) [39]. Considering that CT acylphosphatase does not contain cysteine residues, and that neither peptide residues 13–29 nor peptide residues 20–29 contains tyrosine, the target residue(s) of WRK involved in enzyme inactivation should be restricted to Lys-16, Lys-24 and His-25. Figure 5(A) shows the direct ion-spray mass spectrum of peak 1. It can be seen that it contains mass peaks at 1515.4 m/z $(M^+ + H)^+$, 1262.8 m/z $(M^+ - WRK)$, 758.6 m/z $(M^+ + 2H)^{2+}$ and 632.1 m/z $[(M^+ - WRK) + 2H]^{2+}$. These values fit very well with the WRK adduct of the sequence 20–29 of human CT acylphosphatase, as discussed above. This spectrum also contains two additional mass peaks (1171.1 and 917.1 m/z) which produce information about the WRK target residue. They are fragments of peptide residues 20–29 that fit with the sequence of the free peptide residues 20–26 (H_2N-Val-Phe-Phe-Arg-Lys-His-Thr-CO) (calculated molecular mass, 917.1) and with that of the WRK adduct of the same peptide (calculated mass 1171.4). These fragments contain both Lys-24 and His-25, the amino and imidazole groups respectively which could have reacted with WRK. Nevertheless, we believe that the residue modified by WRK is His-25, since the λ_{max} of the enzyme–WRK adduct agrees more closely with an imidazole–WRK adduct than with a lysine–WRK adduct [39].

Site-directed mutagenesis

The site-directed mutagenesis of a number of residues included in the WRK-labelled peptide named peak 1 (Figure 4) was performed. The WT human CT acylphosphatase and Arg-23 → His, Lys-24 → Gln, His-25 → Gln, His-25 → Phe, and Glu-29 → Gln mutant genes were prepared as described in the Materials and methods section. The major kinetic properties of these enzymes are shown in Table 1. We note that only the Arg-23 → His mutant shows a dramatic loss of activity, the V_{max} was only 0.07% of that of the WT enzyme. Similar results, obtained with three Arg-23 mutants of the MT isoenzyme indicated that this is an essential residue, which is probably involved in substrate binding [40]. Since both MT and organ CT isoenzymes have similar folding [11], we suppose that Arg-23 has a similar role in the catalytic mechanisms of acylphosphatase isoenzymes. Although the K_m and the $K_I(P_i)$ values of Lys-24 → Gln, His-25 → Gln and His-25 → Phe mutants are increased (by 2–3 times), they maintain relatively high V_{max} values (the V_{max} value of the His-25 → Phe mutant is 35% higher than that of the WT enzyme), which suggests that these charged residues do not have important roles in the catalytic pathway of the enzyme. Similar conclusions can be deduced from the kinetic parameters of the Glu-29 → Gln mutant.

We have tested the ability of WRK to inactivate WT and mutant enzymes in order to reveal the residue involved in

WRK–enzyme inactivation. All enzymes were incubated with various WRK concentrations and enzyme activity was monitored for 2 min. Figure 1(B) shows $1/k_{obs}$ against 1/[WRK] with Glu-29 → Gln, His-25 → Gln, His-25 → Phe and Lys-24 → Gln mutant enzymes and of the WT enzyme (the Arg-23 → His mutant was not tested as it had negligible activity). It can be seen that the mutant enzymes agree with the model applied to the WT enzyme, since they show saturation kinetics. We also found that the reactivity of both His-25 → Gln and His-25 → Phe mutants with WRK was dramatically decreased. In fact, we found that at 1.6 mM WRK (pH 6.0, 25 °C) the reactivity of the His-25 → Gln mutant was 7 times lower than that of the WT enzyme (k_{obs}, $7.0 \times 10^{-3} \cdot s^{-1}$ and $1.0 \times 10^{-3} \cdot s^{-1}$ for the WT enzyme and for the His-25 → Gln mutant enzyme respectively); after 2 min incubation, the His-25 → Phe mutant retained almost 100 % activity but the activity of the WT enzyme was approx. 44 %, suggesting that the replacement of His-25 with phenylalanine renders the enzyme almost fully protected against the effects of WRK.

Although WRK is able to modify a total of three enzyme residues (see Figure 2), our findings strongly suggest that His-25 is the most reactive and is the initial target of WRK in the chemical modification of CT acylphosphatase. His-25 is not a conserved residue; all other known acylphosphatases contain tyrosine residues in position 25 of the sequence. The kinetic parameters elicited by the His-25 → Phe mutant, which is more active that the WT enzyme, indicate that the presence of a nucleophilic residue in this position is not necessary for catalysis.

DISCUSSION

We have shown that the plot of inactivation of human CT acylphosphatase activity against increasing levels of modified enzyme follows a straight line up to 70 % residual activity (Figure 2). The extrapolation of this line intercepts the abscissa (100 % inactivation) at a point which indicates WRK-labelling of a single enzyme residue per enzyme molecule. At levels of inactivation greater than 30 % we observed that the slope of the graph changed, suggesting that increasing enzyme modification by WRK is accompanied by the reaction of two additional residues with reactivities lower than that of the first residue.

The main conclusion of this work is that, at relatively low concentrations, WRK interacts specifically with the human CT acylphosphatase isoenzyme and causes enzyme inactivation. The specificity of the WRK–enzyme interaction is probably due to the sulphonate moiety of WRK (a substrate mimetic group), which interacts initially with the anion binding site of the enzyme forming a reversible complex [the dissociation constant (K_I) of this complex is very close to that of the enzyme–P_i complex]. The formation of this complex favours the reaction of the WRK keto ketenimine moiety with the nucleophilic His-25 forming an enamine derivative (the absorption band with λ_{max} at 346 nm of the WRK–enzyme adduct supports the reaction of WRK with an imidazole group [39]). Competitive inhibitors (P_i and ATP), which interact with the anion-binding site of the enzyme, protect the enzyme from inactivation since they hinder the binding of WRK to this site. We emphasize that His-25 → Gln and His-25 → Phe mutants maintain high catalytic activities (Table 1), indicating that His-25, although located near to the active site (it is close to the active-site Arg-23 residue), plays no important role in the catalysis. Thus the observed enzyme inactivation by WRK is probably due to steric hindrance by the reagent, which shields the active site. Alternatively, the covalently-linked WRK may alter acylphosphatase folding locally leading to enzyme inactivation. This latter hypothesis could explain the pronounced reduction in WRK reactivities of both His-25 mutants; in fact, the initial reaction of His-25 with WRK may cause the transposition of the two additional reactive enzyme residues to the solvent (and then to the reagent). Although these residues appear to be protected from WRK attack by P_i, we think that their participation in the enzyme active site is uncertain. Both mutants of His-25, which have high activity levels and clearly contain the two additional residues modified by WRK, are very resistant to WRK inactivation (the His-25 → Phe mutant is almost totally resistant to attack by WRK), suggesting that the two additional residues have no important catalytic roles.

Using protein chemistry to study the initial phase of WRK–enzyme inactivation, we demonstrated that a single enzyme residue was modified. In fact, we purified the WRK adduct from a mixture which contained 70 % residual enzyme activity and we isolated two WRK-labelled peptides (each containing a sole WRK molecule), which originated from the same polypeptide stretch. No other WRK-labelled peak was found in the enzymic fingerprint of the modified protein (Figure 4). In addition, we found that the stoichiometry of the WRK–enzyme adduct, separated from unreacted protein by reverse-phase HPLC, is about 1 mol of WRK/mol of enzyme.

The modification of His-25 during the initial reaction of WRK with CT acylphosphatase is clearly demonstrated by the pronounced lower WRK reactivity of both His-25 mutants and was supported by spectroscopic and MS data.

It was generally thought that the inactivation of enzymes by WRK is caused by the chemical modification of aspartic or glutamic acid residues involved in the active site. On the other hand, our findings clearly demonstrate that the chemical modification of CT acylphosphatase by WRK has no carboxyl targets. About ten years ago, Llamas et al. [39] suggested that the reaction of WRK with the nucleophilic side chains (such as cysteine, histidine, lysine, glutamic and aspartic acid) of several proteins could easily occur, especially at pH values greater than 5.0. On the contrary, several authors have taken the fact that they are inactivated by WRK as evidence for the involvement of acidic amino acids in the active site of several enzymes [27,29,30,34–36,38]. We think that the inactivation of an enzyme by WRK may not be taken as *prima facie* evidence for the presence of an essential carboxy group. In fact, the absorption peak with λ_{max} in the 340–350 nm region, frequently observed in protein–WRK adducts [25,27,29–38], could arise from the reaction of WRK with protein imidazole, thiolate and phenoxide nucleophiles. This is true, since the λ_{max} of the carboxylate enol esters is expected to be centred close to 267 nm [39]. The reaction of WRK with the carboxy group is certainly more specific at pH values in the range 3–5, since the reactivity of carboxy groups in this pH range is greater than that of other nucleophiles [39]. A recent paper, which describes the WRK-inactivation of *Escherichia coli* threonine dehydrogenase, demonstrates that two histidines His-105 and His-205, which are not in the catalytic site, and the essential active-site Cys-38 form WRK adducts [37].

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