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Differential role of four cysteines on the activity of a low M_r phosphotyrosine protein phosphatase

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In this paper we describe the construction of five mutants of a bovine liver low M_r phosphotyrosine protein phosphatase (PTPase) expressed as a fusion protein with the maltose binding protein in *E. coli*. Almost no changes in the kinetic parameters were observed in the fusion protein with respect to the native PTPase. Using oligonucleotide-directed mutagenesis Cys-17, Cys-62 and Cys-145 were converted to Ser while Cys-12 was converted to both Ser and Ala. The kinetic properties of the mutants, using *p*-nitrophenyl phosphate as substrate, were compared with those of the normal protein fused with the maltose binding protein of *E. coli*; both of the Cys-12 mutants showed a complete loss of enzymatic activity while the specific activity of the Cys-17 mutant was greatly decreased (200-fold). The Cys-62 mutant showed a 2.5-fold decrease in specific activity, while the Cys-145 mutant remained almost unchanged. These data confirm the involvement of Cys-12 and Cys-17 in the catalytic site and suggest that Cys-62 and Cys-145 mutations may destabilise the structure of the enzyme.

Acid phosphatase; Phosphotyrosyl protein phosphatase; Fusion protein; Site-directed mutagenesis; Active site

1. INTRODUCTION

The cytosol of many tissues contains a low M_r acid phosphatase, orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2 [1]. This enzyme very efficiently catalyzes the hydrolysis of aryl phosphates, such as *p*-nitrophenyl phosphate and L-P-tyrosine [2,3] and of acyl phosphates such as benzoyl phosphate [4]. The enzyme possesses a very specific phosphotyrosine protein phosphatase activity, which can be demonstrated on several different substrates [5–7]. For this reason the enzyme is now called low M_r phosphotyrosyl protein phosphatase (PTPase). The enzyme from bovine liver consists of a single polypeptide chain of 157 amino acid residues and contains eight cysteines, all in the reduced form [8]. Recently we have expressed the enzyme in *E. coli*: a polynucleotide sequence synthesized on the basis of the protein primary structure was inserted into a bacterial expression vector [9]. A recent experiment of over-expression of the low M_r PTPase in v-erb-B transformed fibroblasts [10] seems to confirm PTPases as possible regulatory elements of cell growth [11].

Several studies have been carried out in an attempt to identify the active site of the enzyme. Inactivation of the enzyme with iodoacetate and protection with the competitive inhibitor, P_i [8], confirmed previous data [3] about the presence of at least a half-cystine residue in or near the active site. Moreover, differential modification of the enzyme by [^{14}C]iodoacetate, in the presence or absence of P_i, showed that Cys-12 and Cys-17 could be the two half-cystine residues involved in the active site of the molecule [8]. More recently Wo et al. [12] were able to trap a phosphoenzyme intermediate during the phosphatase-catalyzed reaction. They also identified the nature of the covalent intermediate as a cysteinyl phosphate. On the other hand, Zhang et al. [13], on the basis of covalent modification experiments, postulated the involvement of Cys-62 and Cys-145 in the active site of the enzyme. In this paper we describe the mutagenesis of low M_r PTPase in four different cysteine residues. The results confirm the involvement of Cys-12 and Cys-17 in the active site of the molecule.

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2. MATERIALS AND METHODS

2.1. *Materials*

Enzymes: Klenow fragment of DNA polymerase I of *E. coli*, T4 DNA polymerase, T4 DNA ligase, polynucleotide kinase and restriction enzymes were obtained from Bio-Rad; *Taq* polymerase from Promega, sequenase from USBC, and factor Xa from Biolabs New England and Sigma.

Vectors: pMALc was from Biolabs New England.

Plasmids: pGEMPTP [10], a derivative of pGEM5Zf$^+$ vector (Promega), harbouring a chemically synthesized polynucleotide sequence coding for bovine liver low M_r PTPase [9], was used.

Bacterials strains: BW313 (*dug*$^-$,*ung*$^-$) was used for mutagenesis [14], and JM101 [15] for pMALc recombinants.

Oligonucleotides were synthesized using a Beckmann System 200A DNA Synthesizer. Amylose resin was obtained from Biolabs New England. Gamma-[^{32}P]ATP (6,000 Ci/mmol) was obtained from NEN.

p-Nitrophenyl phosphate, angiotensin I and L-*P*-tyrosine were obtained from Sigma. Benzoyl phosphate was synthesized by ourselves according to Camici et al. [16]. Horseradish peroxidase-linked mouse anti-rabbit Ig was obtained from Bio-Rad.

2.2. *Methods*

Oligonucleotide-directed mutagenesis with the uridine method, according to Kunkel et al. [17], was performed on the pGEMPTP construct [10]. The TGC codons for Cys-12 and Cys-17 were replaced with the TCC codon for serine or (for Cys-12 only) with the GCC codon for alanine. The TGT codons for Cys-62 and Cys-145 were substituted with the TCT codon for serine. Mutations were confirmed by nucleotide sequence analysis. Polymerase chain reaction experiments for the insertion of the mutated sequences in pMALc vector [18] were performed according to Saiki et al. [19]: fragments spanning the entire coding sequence of normal low M_r PTPase and of the five mutated versions were obtained by amplification. The six different fragments were inserted into the unique *Sta*I and *Hin*dIII sites of the pMALc vector, downstream and in frame with the maltose binding protein coding sequence. After induction with isopropyl thiogalactose, the expression of the six fusion proteins was obtained in *E. coli*; affinity purification on a Sepharose–amylose column [18] allowed us to obtain about 20 mg of fusion proteins per liter of culture.

Phosphorylated angiotensin I was obtained using a partially purified preparation of p40 obtained from calf thymus [20].

SDS-PAGE was performed according to Laemmli et al. [21].

Enzymatic assays for *p*-nitrophenyl phosphate, benzoyl phosphate and L-*P*-tyrosine were performed according, respectively, to Heinrikson et al. [2], Ramponi et al. [22] and Apostol et al. [23].

Western blot analysis was performed according to Tsang et al. [24] using a rabbit anti-PTPase polyclonal antibody and horseradish peroxidase-linked mouse anti-rabbit Ig as the second antibody.

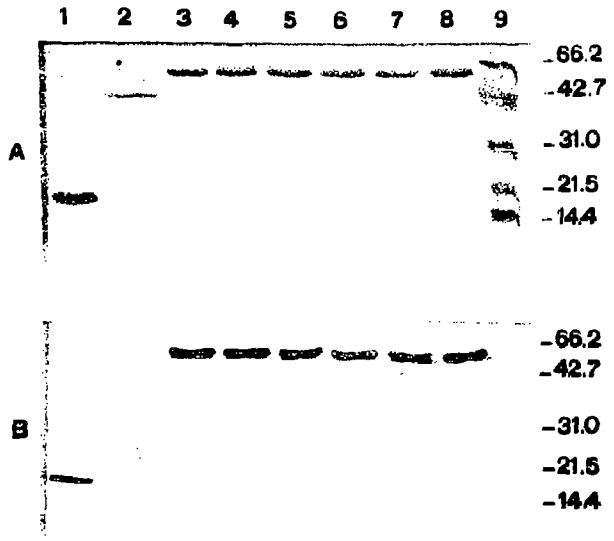

Fig. 1. SDS-PAGE (panel A) and Western blot analysis (panel B) of purified maltose binding protein (MBP) fusion proteins: (1) native PTPase; (2) MBP; (3) MBP-PTPase; (4) MBP-C12A; (5) MBP-C12S; (6) MBP-C17S; (7) MBP-C62S; (8) MBP-C145S; (9) molecular weight standard, with the corresponding values expressed in kDa. Western blot analysis was carried out using rabbit anti-PTPase polyclonal antibody.

3. RESULTS AND DISCUSSION

3.1. *Mutagenesis and expression in the pMALc vector*

In vitro oligonucleotide-directed mutagenesis was performed on the pGEMPTP construct harbouring the low M_r PTPase coding sequence; five mutants were obtained, in which Cys-12 was substituted either with alanine (C12A mutant) or serine (C12S) and Cys-17, Cys-62 and Cys-145 with serine (C17S, C62S and C145S). In order to facilitate purification, sequences coding for the normal and for the five mutated versions of the protein were inserted into pMALc [18]; this vector allows the efficient expression of an heterologous polypeptide in *E. coli* as a fusion protein, where the carboxyl terminus of the maltose binding protein is linked to the amino terminus of the recombinant PTPase.

An SDS-PAGE analysis stained with Coomassie blue and a Western blot analysis of the fusion proteins, purified on an amylose–resin column, are shown in Fig. 1: antibody recognition of PTPase is not impaired by the fusion with maltose binding protein.

The purified fusion proteins were treated with factor Xa, which should separate the low M_r PTPase from the maltose binding protein by cutting the specific Ile-Glu-Gly-Arg sequence present at the joining point of the fusion protein. It has been reported [25] that factor Xa can cleave some proteins which do not contain the specific tetrapeptide; this seems to be the case with low M_r

Table I

Comparison of specific activity and K_m values of native PTPase and PTPase fused to maltose binding protein (MBP) with different substrates

	Specific activity (U/mg)		K_m (mM)	
	Native PTPase	MBP-PTPase	Native PTPase	MBP-PTPase
p-Nitrophenyl phosphate	100.00 ± 1.68	84.60 ± 2.83	0.27 ± 0.03	0.27 ± 0.01
Benzoyl-phosphate (B-P)	52.72 ± 1.35	39.70 ± 1.99	0.86 ± 0.09	1.01 ± 0.07
L-*P*-Tyrosine	33.60 ± 2.99	27.42 ± 1.81		
Tyr-phosphorylated angiotensin I	$1.35 \pm 0.03 \times 10^{-3}$	$1.02 \pm 0.01 \times 10^{-3}$		

Data for K_m values and specific activity were representative of, respectively, three and five experiments. The specific activities of the fusion proteins were calculated considering the M_r of PTPase (29.9% of the M_r of the fusion protein).

PTPase, which is also internally cleaved by factor Xa, rendering purification of native protein very inefficient with this method (data not shown).

3.2. *Biochemical assays with the fusion protein*

It has been reported [26,27] that fusion proteins may maintain the biochemical or biological activity of the native protein. To test this, in a first experiment the catalytic activity of low M_r PTPase purified from bovine liver and of the enzyme obtained as a fusion protein with maltose binding protein, was measured using *p*-nitrophenyl phosphate, L-*P*-tyrosine and Tyr-phosphorylated angiotensin I as substrates (Table I). The specific activity of the fusion protein shows a slight decrease (15–25%) using the three different substrates, whereas the K_m value with *p*-nitrophenyl phosphate is almost identical to that of the native protein. On the basis of these results we can conclude that the fusion of the low M_r PTPase with the maltose binding protein has a moderate influence on the kinetic properties of the enzyme.

3.3. *Biochemical assays with the mutated proteins*

The catalytic activity of the mutated fusion proteins on *p*-nitrophenyl phosphate was determined and compared to the values obtained with the normal PTPase fused with the maltose binding protein (Table II): substitution of Cys-12 either with Ala (C12A) or Ser (C12S) resulted in a complete loss of activity, and therefore determination of kinetic parameters was not possible. In addition, the substitution of Cys-17 with serine (C17S) led to a dramatic reduction (200-fold), although not to a complete loss of the enzymatic activity. The residual activity of C17S mutant, together with the total loss of activity of the C12S mutant suggests a different involvement of these two residues in the catalytic mechanism, in agreement with previous results [8], demonstrating that Cys-12 is more reactive than Cys-17 when compared with the iodoacetate alkylation reaction. On the basis of these results we can postulate a direct involvement of both residues in the catalytic site of the enzyme.

Table II

Specific activity and K_m values of the normal and mutated PTPases fusion proteins using *p*-nitrophenyl phosphate as substrate

	Specific activity PNPP (U/mg)	K_m PNPP (mM)
MBP-PTPase	84.50 ± 2.83	0.27 ± 0.03
MBP-C12A	N.D.	N.D.
MBP-C12S	N.D.	N.D.
MBP-C17S	0.44 ± 0.04	3.58 ± 0.08
MBP-C62S	33.51 ± 2.99	0.83 ± 0.04
MBP-C145S	69.66 ± 1.19	0.81 ± 0.09

Values were obtained as indicated in the legend to Table I. N.D., not detectable.

Recently Zhang et al. [13], on the basis of covalent modification experiments, proposed the involvement of Cys-62 and Cys-145 in the active site of the molecule, together with an unidentified arginine and histidine residue. The two mutants we obtained, C62S and C145S, show only a moderate decrease in specific activity, although to different extents (Table II): with the first mutant we observe a 60% reduction compared to only an 18% reduction with the second mutant. Moreover, the K_m values of these two mutants remain in the same order of magnitude with respect to the normal protein. These results suggest a marginal involvement of Cys-145 and Cys-62 in the enzymatic activity. Alternatively we hypothesize that these two mutations destabilize the structure of the enzyme, causing a slight decrease in the enzymatic activity.

Our results confirm that the active site is a complex structure where several residues can play a differential role in catalysis. On the other hand they confirm our previous findings [8] on the involvement of both Cys-12 and Cys-17 in the active site of the enzyme.

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