Volume 297, number 1,2, 164–166 FEBS 10660 February 1992

Introduction of a free cysteinyl residue at position 68 in the subtilisin Savinase, based on homology with proteinase K

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Received 29 November 1991

Two subfamilies of the subtilisins, distinguished by the presence or absence of a free cysteinyl residue near the essential histidyl residue of the catalytic triad, are known. In order to evaluate the significance of the presence of this -SH group a cysteinyl residue has been introduced by site-directed mutagenesis into the cysteine-free subtilisin-like enzyme from *Bacillus lentus*, i.e. Savinase. The free cysteine affects the enzyme activity only slightly but renders it sensitive to mercurials presumably due to an indirect effect. The results indicate that the -SH group is not involved in catalysis.

Subtilisin; Cysteinyl residue; Mercurial

1. INTRODUCTION

The subtilisins are a well-characterized family of serine endopeptidases secreted by various bacilli and some species of fungi. Proteinase K from *Tritirachium album* [1], thermitase from *Thermoactinomyces vulgaris* [2,3], proteinase B from yeast [4] and the yeast KEX-2-encoded protein [5] constitute a subfamily of the subtilisins which is inhibited by mercurials. They all contain a free cysteinyl residue at an equivalent position in the sequence and the three-dimensional structures of thermitase and proteinase K [6–8] locate it to a position near the essential histidyl residue of the catalytic triad such that it is practically inaccessible to solvents. In the present paper a cysteinyl residue is introduced at position 68 (subtilisin BPN′ numbering [9]) of the cysteine-free subtilisin from *Bacillus lentus*, i.e. Savinase, in order to provide resemblance to the SH-containing subfamily.

2. EXPERIMENTAL

In vitro mutagenesis was performed using a pUC19 subclone of the gene coding for Savinase and the method described by Morinaga et al. [10]. The mutated sequence was re-introduced into the *B. subtilis* expression vector and the mutant Savinase was purified from a 10 l fermentation as previously described [11]. The mutated enzyme was stored frozen in 5 mM MES, 2 mM $Ca(NO_3)_2$, pH 6.5, at −18°C.

The enzyme activity at 25°C towards Suc-Ala-Ala-Pro-Phe-*p*NA (Bachem, Switzerland) was determined as described in [11] using a Perkin Elmer λ7 spectrophotometer. With dimethyl casein as a substrate the proteolytic activity was determined as previously described [12].

Modification of V68C-Savinase with $HgCl_2$ and Ph-Hg-Cl (Merck, Germany) was performed in 0.05 M HEPES, 2 mM $Ca(NO_3)_2$, pH 7.5, using enzyme concentrations of 0.65 and 0.51 μM, respectively. After 30 min incubation at room temperature 10 μl of the reaction mixture was assayed against 0.35 mM Suc-Ala-Ala-Pro-Phe-*p*NA, 0.05 M BICINE, 2 mM $Ca(NO_3)_2$, 5% dimethylformamid, pH 8.5. Modification of V68C-Savinase with CNBr and methyl methanethiolsulfonate was performed in 50 mM HEPES, 2 mM $CaCl_2$, pH 7.5, using an enzyme concentration of 0.54 μM. The modification mixtures contained 50 μM CNBr and 50 μM methyl methanethiolsulfonate, 1% ethanol, respectively.

The thermostability of V68C- and wild-type Savinase was measured by incubating the enzymes in 5 mM MES, 2 mM $CaCl_2$, pH 6.5, at various temperatures, assaying the residual activity as described above.

The enzymes were subjected to thermal analysis by Differential Scanning Calorimetry (DSC) using a Seratam micro DSC apparatus connected to a HP86 computer. The enzyme was diluted to a concentration of 2 mg/ml in 5 mM MES, 2 mM $CaCl_2$, pH 6.5, and the heating rate was 0.5°C/min from 25–90°C.

The content of free cysteine was determined by titration with *para*-hydroxymercuribenzoate in the absence of denaturing agents as previously described [13].

3. RESULTS AND DISCUSSION

The three-dimensional structures of two enzymes belonging to the -SH-containing subfamily of the subtilisins, i.e. proteinase K and thermitase, are known, and both contain a free cysteinyl residue at position 68 (subtilisin BPN′ numbering) which is located behind the

Abbreviations: Savinase, subtilisin 309 from *Bacillus lentus*; MES, 2-[*N*-morpholino]ethane sulfonic acid; V68C Savinase, Savinase with a cysteinyl residue at position 68; Ph-Hg-Cl, phenyl mercuric chloride; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; Suc, succinyl; *p*NA, *p*-nitroanilid; BICINE, *N*,*N*-bis[2-hydroxyethyl]-glycine; SDS, sodium dodecylsulfate; KNPU, kilo novo protease units.

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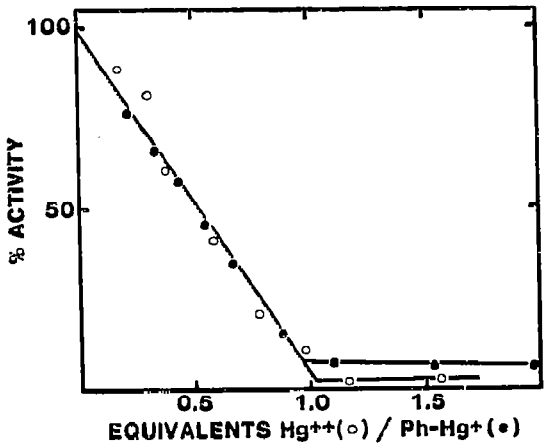

Fig. 1. Inactivation of Savinase with $HgCl_2$ and Ph-Hg-Cl. The enzyme was incubated for 30 min in 0.05 M HEPES, 2 mM $CaNO_3$, pH 7.5, with increasing amounts of mercurials. Subsequently the enzyme was assayed at 25°C towards Suc-Ala-Ala-Pro-Phe-*p*NA as described in section 2.

essential histidyl residue close to the imidazole ring [14] and is practically inaccessible to solvents [7,15]. Addition of one equivalent of Hg^{2+} to proteinase K or thermitase has been shown to reduce the enzymatic activity essentially to zero [16,17] and it is probable that Hg^{2+} binds to the buried cysteinyl residue. This is in accordance with X-ray crystallographic studies of proteinase K and thermitase [7,15].

To study the relationship between the cysteine-containing and the cysteine-free subtilisins a Cys residue was introduced at position 68 of the cysteine-free subtilisin, Savinase. The mutation Val-68 to Cys was accomplished in the structural gene for Savinase by standard procedures. SDS-polyacrylamide gel electrophoresis of the purified enzyme demonstrated its homogeneity and the correct molecular mass (26,700) and titration with *para*-hydroxymercuribenzoate revealed that the enzyme contained 0.94 free -SH group.

To test whether the introduced cysteinyl residue in V68C Savinase is equivalent to the cysteinyl residue in e.g. proteinase K the mutant Savinase was investigated for its sensitivity towards mercurials. By addition of $HgCl_2$ and Ph-Hg-Cl the enzymatic activity towards Suc-Ala-Ala-Pro-Phe-*p*NA decreased linearly with increasing concentrations of mercurials until maximal inactivation was reached at equimolar concentrations (Fig. 1). $HgCl_2$ lowered the activity to 2% while the enzyme modified with Ph-Hg-Cl retained 7% residual activity. The decrease in activity was not due to autolysis or irreversible denaturation of the enzyme since incubation of the Hg^{2+}-modified enzyme with 0.1 M mercaptoethanol fully restored the activity of the enzyme within 10 minutes. These mercury compounds (1.5 equivalents) did not affect the activity of wild-type Savinase.

In contrast, the two non-bulky reagents, CNBr and methyl methanethiolsulfonate, known to react with sulfhydryl groups [18,19], did not affect the activity towards Suc-Ala-Ala-Pro-Phe-*p*NA, indicating that neither cyanylation nor thioalkylation took place. From the structural data it is known that position 68 of Savinase is buried and inaccessible to solvents [20] and therefore it is peculiar why the bulky Ph-HgCl reacts rapidly and stoichiometrically with the -SH group while the non-bulky SH-reagents do not. A possible explanation is that the Hg-containing compounds are able to increase the flexibility of the region around position 68.

As judged by the inhibition criteria V68C Savinase resembles the -SH-containing subfamily of the subtilisins. It has been suggested that the free -SH group in proteinase K and thermitase influences the activity of the enzymes [14] and furthermore, it has been demonstrated that a sulfur-containing amino acid side chain at position 222 of subtilisin, in the vicinity of the catalytic triad, influences catalysis [11,12]. In order to further explore the effect of a Cys residue at position 68 the kinetic parameters for hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA were determined and it turned out that k_{cat} and K_m were only slightly affected compared to the wild-type enzyme (Table I). With dimethyl casein as a substrate the activities of wild-type and V68C Savinase were also comparable, with the mutant exhibiting a relative activity of 120% (Table I).

Table I

Kinetic parameters and proteolytic activity of Savinase and V68C Savinase

Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	Activity (KNPU/A_{280})
Savinase	7,800	1.8	4,300	328
V68C Savinase	7,900	2.2	3,600	390

The kinetic parameters and k_{cat}, K_m and k_{cat}/K_m for the hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*NA and the proteolytic activity, using dimethylcasein as substrate, were determined for Savinase and V68C Savinase. The proteolytic activity is expressed as specific activity in arbitrary units (KNPU) divided by protein concentration, measured by absorbance at 280 nm, relating the activity of the enzymes to the activity of a subtilisin standard.

The stability of wild-type Savinase and V68C Savinase at 65 and 70°C was determined. The mutant was inactivated with $t_{1/2}$= 30 min and $t_{1/2}$= 4 min, respectively, while the corresponding values for wild-type Savinase were $t_{1/2}$ = 96 min at $t_{1/2}$ = 8.5 min, respectively. Differential Scanning Calorimetry of the mutant enzyme showed a destabilization of 2°C compared to wild-type Savinase. The thermal stability of Savinase is thus slightly reduced by the Val-68-to-Cys mutation and therefore it is not very likely that the free cysteinyl residue contributes to the thermal stability of proteinase K and thermitase.

The results above demonstrate that a cysteinyl

residue introduced at position 68 in Savinase, close to the catalytic triad [20], has very little effect on the activity of the enzyme but renders it sensitive to mercury compounds. Therefore, it is probable that the free Cys of the -SH-containing family of subtilisins is not an essential residue; the effect of Hg^{2+} is indirect, presumably through interaction with the essential His [7]. Carboxypeptidase Y from yeast, utilizing the catalytic mechanism of the serine endopeptidases [22], contains a similar single free -SH group. This cysteinyl residue also appears not to be essential since neither its replacement with other residues by site-directed mutagenesis [23] not chemical modifications [18,24] abolish the activity. However, it still remains a mystery why a reactive cysteinyl residue is situated close to the active site in otherwise unrelated enzymes.

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