Rat thimet oligopeptidase: large-scale expression in *Escherichia coli* and characterization of the recombinant enzyme

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The coding sequence for rat testis thimet oligopeptidase (TOP) (EC 3.4.24.15) was placed under the control of the T7 polymerase/promoter system. Cultures of *Escherichia coli* transfected with the resulting plasmid expressed the enzyme as a soluble cytoplasmic protein. Medium-scale cultures allowed isolation of the enzyme in quantities of tens of milligrams. The availability of the recombinant enzyme permitted the determination of such

chemical properties as ϵ_{280} (48960), zinc content (2 atom/molecule) and available thiol content (8–10/molecule) for TOP. The recombinant enzyme showed the catalytic activities previously reported for the naturally occurring enzyme, so that we can now conclude with confidence that these are all due to TOP and there is no need to postulate the existence of separate ‘Pz-peptidase’ or ‘endo-oligopeptidase A’ enzymes.

INTRODUCTION

Thimet oligopeptidase (EC 3.4.24.15; TOP) is a metalloendopeptidase that shows thiol dependence in its activity and is normally restricted to action on peptides in the range 5–17 residues [1,2]. Earlier literature on the enzyme, including the several names under which it has previously been known, has been reviewed [3,4]. TOP is located in the soluble phase of the cytoplasm and is also associated with organelles that are probably endosomes [5]. There is evidence that in yeast a homologue of TOP participates in the cytosolic degradation of oligopeptides [6], and the mammalian enzyme may well have a similar function. The metabolism of oligopeptides in the cytoplasm is currently of interest as a stage in the pathway of protein degradation that is initiated by the proteasome and ATP/ubiquitin-dependent proteolytic systems. This pathway is responsible for much of the turnover of short-lived proteins in the cell [7] and supplies antigenic peptides to the major histocompatibility class I complexes [8].

Studies of the small quantities of TOP that have been isolated from natural sources have yielded essentially all of the information available to date on the physicochemical properties and enzymology of the enzyme [4]. Questions remained, however, that can now be answered by use of TOP expressed in a bacterium. For example, the larger quantities of the recombinant protein allow the determination of such basic physicochemical properties of TOP as molar absorbance coefficient and the content of metal and thiol groups. The availability of the recombinant enzyme also allows us to resolve a long-standing question about the identity of TOP. It has been our view that TOP is responsible for activities described earlier as ‘Pz-peptidase’ [9] and ‘endo-oligopeptidase A’ [10]. This view has been supported by much further work with highly purified preparations of the enzyme (e.g. [1,11]), but there have also been a few contrary reports. It has been suggested that some of the activities attributed to TOP are really due to contamination by a

separate and distinct endo-oligopeptidase A [12,13]. Substrates particularly mentioned as specific for endo-oligopeptidase A are bradykinin, dynorphin A^{1–8} and a synthetic substrate analogue of dynorphin A^{1–7}, *o*-aminobenzoyl-Gly-Gly-Phe-Leu-Arg-Arg-N-(2,4-dinitrophenyl)ethylenediamine [13,14]. Since neither TOP nor endo-oligopeptidase A is detectable in wild-type *Escherichia coli*, the specificity of TOP expressed in the bacterium can be expected to reveal unambiguously the specificity of this enzyme, and thus resolve the controversy.

Although TOP does not hydrolyse most substrates of more than 17 amino acid residues that have been tested [1], there have been proposals that the enzyme may act on such larger molecules as Alzheimer’s amyloid precursor protein [15] and farnesylated proteins [16]. Again, these questions may be easier to resolve with the recombinant enzyme, free from other eukaryotic peptidases.

A longer-term objective of work with recombinant TOP (rTOP) is a better understanding of the catalytic mechanism of the enzyme and the structural basis of the restriction of substrate molecular size. The availability of large quantities of the enzyme are allowing attempts at crystallization for the determination of the three-dimensional structure. Moreover, once the active, wild-type enzyme can be expressed, the opportunity exists for site-directed mutagenesis to probe the functions of those amino acid residues that are currently thought, on the basis of purely circumstantial evidence, to be of catalytic importance.

Rat TOP was cloned by Pierotti et al. [17] and cDNA (plasmid p44) was made available to the present authors by Drs. J. L. Roberts and M. J. Glucksman (Mount Sinai School of Medicine, NY, U.S.A.). We made some corrections to the previously published sequence and showed that TOP is related to ‘angiotensin II-binding protein’ [18]. More recently, we have demonstrated that the angiotensin-binding protein is neurolysin (EC 3.4.24.16) [19].

Small-scale expression of TOP has been reported [17,20], but we sought an expression system that would reproducibly yield large amounts of TOP, and which in combination with a system

Abbreviations used: DTT, dithiothreitol; QF01, 2,4-dinitrophenyl-Pro-Leu-Gly-Pro-Trp-D-Lys; QF02, 7-methoxycoumarin-3-carboxylyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl); Pz, N^ε-(4-phenylazo)benzyloxycarbonyl; rTOP, recombinant rat thimet oligopeptidase; TOP, thimet oligopeptidase (EC 3.4.24.15).

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for site-directed-mutagenesis would allow the efficient expression of mutant enzymes. We here describe such a system and the purification of the recombinant enzyme to homogeneity. The recombinant enzyme has been used to answer some of the questions raised above.

MATERIALS and METHODS

Materials

TOP from rat testis was obtained as described by Dando et al. [1]. 2,4-Dinitrophenyl-Pro-Leu-Gly-Pro-Trp-D-Lys (QF01) and 7-methoxycoumarin-3-carboxyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl) (QF02) were provided by Dr C. G. Knight (of this laboratory) or purchased from Calbiochem/Novabiochem. Iodoacetamide, iodoacetic acid, *N*-ethylmaleimide and *N*-phenylmaleimide were purchased from Sigma. *N*-Phenylmaleimide was recrystallized from cyclohexane and the other compounds were used as supplied.

DNA manipulation, bacterial strains and plasmids

The *E. coli* strain TG1 [22] which was used for all subcloning reactions was the gift of Dr C. F. Hawkins (Department of Biochemistry, University of Cambridge, U.K.). It was maintained on minimal medium, supplemented with 0.1 mM thiamine hydrochloride to maintain selection for the F' episome. The *E. coli* strain BL21 DE3 (pLysS) used for expression of TOP has been described [23]. *E. coli* strains were grown at 37 °C in 2TY broth (1% yeast extract/1% tryptone/0.5% NaCl) unless otherwise noted. The plasmid pTZ18r was obtained from Pharmacia and the expression plasmid pT7-7 [24] was the gift of Dr P. Caffrey (Department of Biochemistry, University of Cambridge, U.K.).

All DNA manipulations, including the isolation of DNA from agarose gels, DNA ligation and the transformation of *E. coli* were as previously described [25] or were carried out by standard procedures [22]. DNA sequence analysis was done using the dideoxy-chain-termination method [26] in the Sequenase system (US Biochemicals). Site-directed mutagenesis was accomplished by the uracil-mediated method described [27], as supplied in kit form by Pharmacia. Oligonucleotides were purchased HPLC-purified and 5'-phosphorylated from Cruachem (Glasgow, Scotland, U.K.) and used as supplied.

Construction of the pEXTOP expression vector

The expression vector pEXTOP (Figure 1) was constructed from the plasmid p44 that contained the coding sequence for rat TOP as a full-length cDNA isolated from a rat testis cDNA library [17,18]. The expression system used was based on one developed previously for a prokaryotic enzyme [25], employing the highly efficient T7 polymerase/promoter system developed by Studier et al. [23]. The 5'-untranslated region of the TOP cDNA was removed by the insertion of a restriction site for endonuclease *NdeI* spanning the start codon of the TOP gene. In order not to run the risk of introducing mutations into the TOP cDNA at locations other than those desired, site-directed mutagenesis was performed on a subclone (pSac 160) of the p44 plasmid which contained the 5'-untranslated region and 105 bases of the TOP gene [17]. The plasmid pSac 160 was created by restriction digestion of p44 with *SacI* and the isolation and re-ligation of the *SacI* fragment into *SacI*-digested pTZ18r. The resulting plasmid was used to transform *E. coli* CJ236 [27] for the preparation of single-stranded, uracil-containing DNA, with the use of helper phage infection. The insertion of the *NdeI* site was accomplished

Figure 1 The expression plasmid pEXTOP

The plasmid (4.6 kbp) was constructed by insertion of the 2.2 kbp *NdeI*-*HindIII* fragment derived from the plasmid pNDETOP into the vector pT7-7 (see text). The arrow in the sequence denotes the translation start site of the TOP gene.

with the 21-base oligonucleotide 5'-GCCTGCCCATATGAA-GCCCCC-3' (mis-matched bases underlined) as a mutagenic primer on the single-stranded, uracil-DNA isolated from *E. coli* CJ 236/pSac 160, to create the plasmid pSac 160-N.

The *SacI* insert containing the *NdeI* site was re-sequenced to confirm that no other mutations were introduced and that the *NdeI* site was introduced at the correct place spanning the start codon of the TOP gene. The *SacI* fragment from pSac 160-N was isolated and subcloned back into p44 to create the plasmid pNDETOP, which is the re-constituted p44 bearing an *NdeI* site spanning the start codon. The TOP coding sequence was isolated as an *NdeI*-*HindIII* fragment from the plasmid pNDETOP, and subcloned into the vector pT7-7 to create the expression vector pEXTOP (Figure 1), in which the TOP sequence is orientated for optimized transcription and translation initiation from phage T7 signals encoded by the vector [23,24]. The plasmid pEXTOP was used to transform *E. coli* strain BL21 DE3 (pLysS). Transformants were selected by resistance to chloramphenicol and ampicillin.

Induction of the expression of TOP in *E. coli*

In order to enrich for ampicillin-resistant and hence plasmid-bearing clones, the *E. coli* BL21 DE3 (pLysS)/pEXTOP cells were streaked on to 2TY plates containing chloramphenicol (33 µg/ml) and ampicillin (100 µg/ml) and grown overnight at 37 °C. A single colony from this plate was inoculated into 3 ml of 2TY medium with chloramphenicol (33 µg/ml) and ampicillin (100 µg/ml) and grown until the culture was becoming turbid ($D_{600} = 0.05$). The cells were then streaked on to a fresh 2TY plate with antibiotics as above and incubated for a further 12 h at 37 °C. A single colony from this plate was used to inoculate 100 ml of 2TY and this was incubated overnight at 37 °C. The overnight culture was then centrifuged, washed in 200 ml of fresh 2TY medium and re-suspended in 20 ml of 2TY medium. These cells served as inoculum for the large-scale cultures used for isolation of the enzyme. In each experiment, 200 µl of the overnight culture was used to inoculate a 1 litre culture in Terrific Broth [22] and grown until the D_{600} was > 1.5. At this point the production of TOP was induced by the addition of isopropyl β-D-thiogalactopyranoside (final concentration 0.4 mM) and cells were grown for 16 h at 37 °C before harvesting.

Protein analyses

Protein was determined with Coomassie Brilliant Blue by use of the Bio-Rad kit. SDS/PAGE was as described [28]. Automated

N-terminal sequence analysis was done by Dr R. A. Harrison (MRC Molecular Immunopathology Unit, Cambridge, U.K.).

Enzyme assays

The activity of TOP was generally assayed with QF02 [29], but QF01 was used as stated; the conditions for both substrates were as described by Barrett et al. [4]. The hydrolysis of other peptides was followed by HPLC [1].

Purification of rTOP

All steps in the purification of rTOP were at 4 °C. Buffer A contained Tris/HCl at the given molarity (of HCl), pH 7.8, with the addition of 0.05 % Brij-35, 5 mM 2-mercaptoethanol and 100 μ M ZnCl₂, which were found to stabilize the enzyme.

Extraction

Cells from 20 l of culture were collected by centrifugation (4000 g), and washed twice in 50 mM Tris/HCl buffer, pH 7.8, containing 100 mM NaCl, and twice more in buffer alone. The cell pellet (200 g) was frozen at -70 °C and then thawed at 35 °C, with the addition of 3.2 l of 50 mM buffer A. The cell suspension was homogenized in a Waring blender (30 s), and frozen and thawed once more. The mixture was prepared to contain 0.1 mg of protamine sulphate/ml and centrifuged at 10000 g.

Batchwise DEAE-cellulose

The supernatant was diluted with 1.5 vols. of water and stirred overnight at 4 °C with 400 g of damp DE-52 DEAE-cellulose (Whatman) that had been equilibrated with 20 mM buffer A. The ion-exchanger was collected by centrifugation (15 min at 3000 g) and washed three times with 20 mM buffer A. The enzyme was then eluted by two treatments of the exchanger with 0.4 M NaCl in the same buffer (total volume of washings about 1 litre).

Ammonium sulphate fractionation

The solution was made 1.4 M in ammonium sulphate by addition of the solid, and after 30 min the precipitate was removed by centrifugation (8000 g) and discarded. The supernatant was made 2.4 M in ammonium sulphate and the precipitate, collected as above, was dissolved in the minimum volume of 50 mM buffer A and dialysed against the same buffer overnight.

DEAE-cellulose chromatography

The crude enzyme sample was run on to a bed (2.5 $\times$ 60 cm) of DE-52 equilibrated with 20 mM Buffer A. The column was washed with the 20 mM buffer and then eluted with a gradient to 0.3 M NaCl in the buffer. Active fractions were combined. The enzyme was concentrated by precipitation from 2.4 M ammonium sulphate and the pellet redissolved in 20 mM Buffer A modified by the omission of Brij-35 and the addition of 1.25 M ammonium sulphate.

Pentyl-agarose chromatography

The solution in 1.25 M ammonium sulphate was run on to a 100 ml bed of pentyl-agarose (Sigma, P5393). The column was washed with two bed vols. of 1.25 M ammonium sulphate and then eluted with a gradient descending from 1.25 M to zero

ammonium sulphate in the buffer. Active fractions were combined and dialysed into 20 mM Buffer A.

Resource Q chromatography

The preparation was divided into two equal parts to be run separately on the Resource Q column (Pharmacia 17-1179-01). Elution was with a gradient to 0.2 M buffer A. Fractions were assayed and also run on SDS/PAGE, and those of specific activity 3–6 units/mg that showed a single protein band were combined as the final product. The pure enzyme was stored as a solution of at least 5 mg/ml in 20 mM buffer A plus 100 mM NaCl and 0.1 % NaN₃ at 4 °C.

Determination of molar absorbance coefficient

A solution of rTOP (about 5 mg) was dialysed against three changes of 1 % acetic acid during 24 h, and triplicate determinations of the A_{280} of the retentate were made. Three samples of the retentate, each containing about 1.0 mg of rTOP, were transferred to weighed tubes, freeze-dried and further dried at 50 °C *in vacuo* overnight. Samples of identical volume of the diffusate were treated in parallel and all the tubes were reweighed. The tubes that had contained diffusate showed no detectable change in weight. From the mean values of A_{280} and dry weight, the $A_{280}^{1\%}$ and molar absorbance coefficient were calculated.

Determination of zinc

Zinc was quantified by Dr. J. G. Williams of the NERC ICP-MS facility (Ascot, Berkshire, UK) by use of inductively-coupled plasma mass spectrometry.

Determination of thiol content

Tris/HCl buffer (50 mM), pH 7.9, and the same buffer containing 250 mM NaCl, were degassed under vacuum and bubbled with N₂ before use, and the Mono Q HR5/5 column of the Pharmacia FPLC apparatus was pre-equilibrated with the low-salt buffer. TOP in 50 mM Tris/HCl, pH 7.9, was treated with 5 mM dithiothreitol (DTT) (15 min, 20 °C) and separated from the reducing agent by chromatography on the Mono Q column with a gradient to 250 mM NaCl. Samples of the protein peak were subjected to spectrophotometric titrations with 5,5'-dithiobis-(2-nitrobenzoic acid) and 2,2'-dipyridyl disulphide in the presence of 1 % SDS [30,31].

RESULTS and DISCUSSION

TOP expressed in the *E. coli* cells of strain BL21 DE3 remained in the soluble fraction after ultracentrifugation, but the level of expression was not as high as for bacterial proteins previously expressed in this system [25]. This may result from the codon-usage of the rat cDNA or a secondary-structural block to efficient start of translation at the chimeric T7-rat cDNA junction. TOP continued to be expressed during many hours of continuous culture however, and since the protein had no apparent effect on bacterial growth, cultures were routinely maintained for 16 h after induction to allow accumulation of the recombinant enzyme.

Purification of rTOP

The progress of purification of rTOP is summarized in Table 1 and Figure 2. In preliminary experiments (results not shown) it

Table 1 Purification of rTOP

The values are means from two preparations done as described in the Materials and methods section.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Cell extract	17 600	688	0.039	(1)	(100)
DEAE-cellulose (batch)	9900	428	0.043	1.10	62
Ammonium sulphate	4200	403	0.096	2.46	59
DEAE-cellulose (column)	412	390	0.947	24.3	57
Pentyl-agarose	109	297	2.72	69.7	43
Resource Q	41	157	3.83	98.2	23

Figure 2 SDS/polyacrylamide gel electrophoresis showing the purification of rTOP from the *E. coli* cell lysate

Samples applied to the 10% polyacrylamide gel reflected the results of purification in steps as follows: (a) treatment of cell lysate with protamine sulphate; (b) ammonium sulphate fractionation; (c) DEAE-cellulose chromatography; (d) pentyl-agarose; and (e) Resource Q.

was found that inclusion of 100 μM ZnCl_2 in the buffers used in the purification markedly stabilized the enzyme during the procedure, contributing to the high overall recovery.

SDS/PAGE (Figure 2) shows that TOP became the major protein after the DEAE-cellulose chromatography step. A 100-fold purification gave 40 mg of homogeneous enzyme preparation, on this scale, with 20% yield.

The rTOP was run alongside the enzyme from rat testis in SDS/PAGE and the gel was blotted on to nitrocellulose and developed with sheep anti-(rat TOP) serum as described [1]. It was found that the two forms of the enzyme were indistinguishable in apparent M_r , and in immunoreactivity (results not shown). The two forms of TOP were also blotted on to polyvinylidene difluoride membrane for automated determination of N-terminal sequences, and the results were: (M)KPPAACAG- (rat testis) and KPPAAXA- (recombinant). Residue Met-1 was partially absent from the testis enzyme preparation and completely absent from the rTOP.

The findings that the recombinant enzyme was similar in activity to the natural form and that both contained the full-length N-terminus, suggest that TOP is not synthesized as an

inactive precursor, as many endopeptidases of all catalytic classes are.

Molar absorbance coefficient

Quantities of TOP isolated from natural sources have not been sufficient to permit the determination of the molar absorbance coefficient of the protein. Triplicate determinations were made as described in the Materials and methods section. From the results, it was calculated that $A_{280}^{1\%} = 6.25$ for rTOP, and on the basis of the molecular mass of 78343 (from the amino acid composition, with no disulphide bonds), $\epsilon_{280} = 48960$.

Zinc content

Solutions of TOP of known concentration were analysed for zinc as described in the Experimental section. From the results, it was calculated that rTOP contains 1.8 g-atom/mole of zinc, indicating that most molecules contain two atoms of the metal.

It has previously been found that the apoenzyme of TOP is efficiently reactivated by Zn^{2+} [32] and the analysis of a small amount of enzyme purified from rat testis gave a value of 1.7–2.0 g-atom of zinc/mole of enzyme [33]. Matrix metalloproteinases also have been found to contain two atoms of zinc per molecule [34].

Thiol content

The thiol content of TOP was determined spectrophotometrically, as described above, with both 5,5'-dithiobis-(2-nitrobenzoic acid) and 2,2'-dipyridyl disulphide as titrants. In several experiments, 8–10 thiol groups were detected per molecule of the enzyme denatured in 1% SDS. The amino acid composition calculated from the sequence of rat TOP [4] shows 15 cysteine residues per mole. Our data do not exclude the possibility that some of these form disulphide bonds, but these are not expected in a cytosolic enzyme and many proteins contain cysteine side chains that are unreactive even after denaturation [31].

Catalytic activity

The rTOP was compared with the enzyme from rat testis with regard to K_m for the hydrolysis of two synthetic substrates. Values for rTOP and the rat testis enzyme were 12 and 16 μM respectively for QF01, and 23 and 34 μM for QF02.

Preparations of rTOP appearing homogeneous in SDS/PAGE ranged from 3 to 6 units/mg in specific activity. This agreed closely with expectation, since the enzyme purified from rat testis was obtained at 6.5 units/mg, but the specific activity soon fell back to 3 units/mg in storage [1].

The sites of cleavage of eight substrates by rTOP were determined by HPLC analysis. The substrates were Pz-Pro-Leu-

Gly-Pro-D-Arg, QF01, QF02, *N*^z-benzoyl-Gly-Ala-Ala-Phe-*p*-aminobenzoate, bradykinin, dynorphin A¹⁻⁸, *o*-aminobenzoyl-Gly-Gly-Phe-Leu-Arg-Arg-*N*-(2,4-dinitrophenyl)ethylenediamine and neurotensin. The cleavage positions were exactly as found previously for the hydrolysis of these substrates by the natural rat and human enzymes [1]. Also, the rates of hydrolysis of the substrates were of a similar order to those obtained with the natural rat enzyme.

In the argument for a distinct endo-oligopeptidase A, it has been suggested that TOP lacks the ability to cleave enkephalin-containing peptides at the Leu-Arg bond to release enkephalin, and to cleave bradykinin at the Phe-Ser bond [12,13]. These activities were seen with highly purified preparations of TOP from rat testis and human red cells [1,11], but perhaps it could still have been argued that the purified preparations of TOP were contaminated with endo-oligopeptidase A. Even that remote possibility is now excluded by the finding that rTOP cleaves the substrates suggested to be selective for endo-oligopeptidase A, just as the natural enzyme did.

Thiol dependence

One of the distinctive characteristics of TOP is its activation by thiol compounds at low concentrations and inhibition at higher concentrations [32]. Rat testis TOP and rTOP were both dialysed overnight against buffer lacking thiol compounds and assayed for activity directly or with the addition of with 0.1 or 1.0 mM DTT. Both preparations were activated to about 200% at 0.1 mM DTT and inhibited to about 50% (of the value in 0.1 mM DTT) at 1 mM DTT.

Effects of alkylating agents

rTOP was fully activated with DTT in a small volume of solution and was then diluted into a large volume of 1 mM solution of alkylating agent in large excess over the total thiol. The alkylating agents used were iodoacetate, iodoacetamide, *N*-ethylmaleimide and *N*-phenylmaleimide. Assays were done 5 and 10 min later and gave similar results, indicating that the reactions with the alkylating agents had gone to completion. Activities (means of duplicates) relative to the control without alkylating agent were: iodoacetate (116%), iodoacetamide (96%), *N*-ethylmaleimide (46%) and *N*-phenylmaleimide (12%).

It was concluded that alkylation of all readily reactive thiol groups of TOP affects activity in a way that depends upon the nature of the alkylating groups. The small, negatively charged carboxymethyl groups cause an increase in activity under the assay conditions whereas the progressively larger and more hydrophobic carboxamidomethyl, ethylsuccinimido and phenylsuccinimido substituents cause increasing degrees of inhibition.

Other inhibitors

rTOP was dialysed into 50 mM Tris/HCl buffer, pH 7.8, containing 5 mM 2-mercaptoethanol. Values of *K_i* were determined for rTOP and testis TOP with Cpp-Ala-Ala-Phe-*p*-aminobenzoate (58 and 30 nM) and dynorphin A¹⁻¹³ (82 and 48 nM). Thus, the values for rTOP were slightly higher than those for the natural enzyme, by a factor of < 2.

CONCLUSIONS

The rTOP was essentially indistinguishable from the enzyme

purified from rat testis by the criteria we have used, apart from slight differences in *K_m* values for QF01 and QF02.

The achievement of large-scale expression and purification of TOP opens the way to further protein chemistry, attempts at crystallization for the determination of three-dimensional structure and site-directed mutagenesis to explore the role of specific amino acid residues in the thiol-dependent oligopeptidase activity of this enzyme.

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