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J. Bacteriol. **1995, 177(19):5561.**

JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5561–5566
0021-9193/95/$04.00+0

Vol. 177, No. 19

Molecular Cloning, Nucleotide Sequence, and Expression of a Carboxypeptidase-Encoding Gene from the Archaebacterium *Sulfolobus solfataricus*

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Received 24 March 1995/Accepted 18 July 1995

Mammalian metallocarboxypeptidases play key roles in major biological processes, such as digestive-protein degradation and specific proteolytic processing. A *Sulfolobus solfataricus* gene (*cpsA*) encoding a recently described zinc carboxypeptidase with an unusually broad substrate specificity was cloned, sequenced, and expressed in *Escherichia coli*. Despite the lack of overall sequence homology with known carboxypeptidases, seven homology blocks, including the Zn-coordinating and catalytic residues, were identified by multiple alignment with carboxypeptidases A, B, and T. *S. solfataricus* carboxypeptidase expressed in *E. coli* was found to be enzymatically active, and both its substrate specificity and thermostability were comparable to those of the purified *S. solfataricus* enzyme.

Mammalian metallocarboxypeptidases play key roles in major biological processes, ranging from digestive-protein degradation, as effected by carboxypeptidases A and B (9, 13), to specific proteolytic processing, as in the maturation of biologically active peptides. This latter is the role, for instance, of carboxypeptidase N and enkephalin convertase (10, 17).

Little is known, however, regarding microbial carboxypeptidases. Recent studies led to the cloning and crystallization of a metallocarboxypeptidase from *Thermoactinomyces vulgaris* (27, 29), which was shown to possess a dual substrate specificity, namely, the capacity to cleave both hydrophobic and basic amino acid residues, combining the activities of carboxypeptidases A and B, respectively. Sequence comparison revealed a sequence similarity of approximately 30% and a three-dimensional structure very similar to those of both of these mammalian enzymes (27, 29). Another microbial carboxypeptidase with broad substrate specificity was also isolated recently from the thermophilic eubacterium *Thermus aquaticus* (16). However, no sequence data are so far available for this enzyme.

The lack of any knowledge regarding archaebacterial carboxypeptidases led us to purify and characterize one such enzyme from *Sulfolobus solfataricus* (3), an extreme thermoacidophilic archaebacterium, isolated from volcanic hot springs, that grows optimally at 87°C (7). We found that this protein is a zinc metalloprotease endowed with a unique substrate specificity in that it could release basic, acidic, aromatic, and, to a lesser extent, aliphatic amino acids from artificial substrates. Also, it could withstand temperatures of up to 85°C and some organic solvents at up to 40°C. Furthermore, we found that salt bridges and the zinc ion play major roles in the kinetic thermal stabilization of this molecule (30). Here, we report the molecular cloning and complete nucleotide sequence of the carboxypeptidase-encoding gene. We show that despite the lack of overall sequence homology with known carboxypeptidases, seven blocks of homology with known metallocarboxypeptidases, spanning the Zn-coordinating histidines, can be identified. We also show that *S. solfataricus* carboxypeptidase expressed in *Escherichia coli* is enzymatically active and that both its substrate specificity and thermostability are comparable to those of the purified *S. solfataricus* enzyme.

MATERIALS AND METHODS

Enzymology. *S. solfataricus* carboxypeptidase was purified as previously described (3). Carboxypeptidase activity was assayed as previously reported (3) by a cadmium-ninhydrin colorimetric method (8). The enzyme was incubated at 60°C in 120 μl of 0.1 M potassium MES (potassium morpholineethanesulfonic acid) (pH 6.5) with, unless otherwise stated, 2 mM benzyloxycarbonyl-Arg as a substrate. After suitable incubation times, 0.9 ml of cadmium-ninhydrin reagent was added, the mixture was heated for 5 min at 84°C, and the A_{505} was read. One unit of enzyme activity is defined as the amount which hydrolyzes 1 μmol of substrate per min under the above-mentioned conditions. For thermal stability

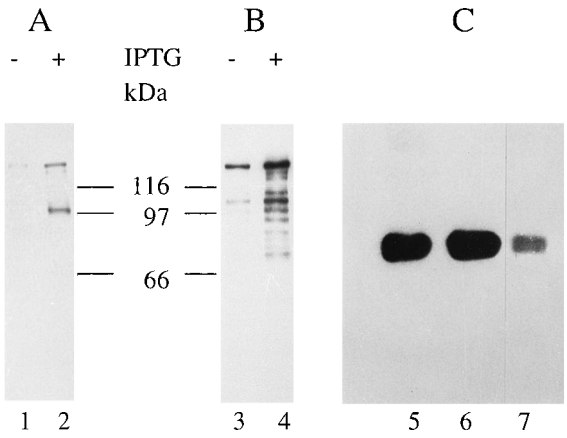

FIG. 1. Immunoblot analysis of Y1089/λCP14A lysogen, grown either in the absence (−) or in the presence (+) of 1 mM IPTG, probed with either anti-β-galactosidase (A) or anticarboxypeptidase (B) antibodies. (C) Specificity of anticarboxypeptidase antibodies purified on λCP14A protein and probed on an *S. solfataricus* crude extract (5 μg) (lane 7). Lanes 5 and 6, purified carboxypeptidase (50 ng) and an *S. solfataricus* crude extract (5 μg), respectively, probed with the affinity-purified antibodies used in the immunological screening.

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                    Met Asp Leu Val Glu Lys Leu Lys Asn Asp Val Arg Glu Ile Glu     15
          TTC GGT CTT ATG GAT TTA GTT GAG AAG TTA AAA AAT GAC GTA AGA GAA ATA GAG    54

Asp Trp Ile Ile Gln Ile Arg Arg Lys Ile His Glu Tyr Pro Glu Leu Ser Tyr Lys Glu    35
GAC TGG ATA ATT CAA ATT AGA AGG AAA ATC CAT GAG TAT CCG GAA CTT TCC TAC AAG GAG   114

Tyr Asn Thr Ser Lys Leu Val Ala Glu Thr Leu Arg Lys Leu Gly Val Glu Val Glu Glu    55
TAT AAC ACC TCT AAA CTA GTA GCG GAA ACG TTA AGG AAA TTG GGA GTA GAA GTG GAA GAA   174

Gly Val Gly Leu Pro Thr Ala Val Val Gly Lys Ile Arg Gly Ser Lys Pro Gly Lys Thr    75
GGC GTT GGA TTA CCC ACA GCA GTG GTT GGT AAG ATT AGG GGA AGT AAA CCA GGA AAG ACT   234

Val Ala Leu Arg Ala Asp Met Asp Ala Leu Pro Val Glu Glu Asn Thr Asp Leu Glu Phe    95
GTT GCT TTG AGA GCT GAT ATG GAT GCC CTT CCG GTA GAG GAG AAC ACT GAT CTA GAA TTT   294

Lys Ser Lys Val Lys Gly Val Met His Ala Cys Gly His Asp Thr His Val Ala Met Leu   115
AAA TCC AAA GTT AAG GGA GTA ATG CAC GCA TGT GGT CAT GAT ACT CAC GTA GCA ATG CTC   354

Leu Gly Gly Ala Tyr Leu Leu Val Lys Asn Lys Asp Leu Ile Ser Gly Glu Ile Arg Leu   135
TTA GGT GGA GCT TAT CTG TTA GTT AAG AAT AAA GAT TTA ATC AGT GGT GAA ATT AGG TTA   414

Ile Phe Gln Pro Ala Glu Glu Asp Gly Gly Leu Gly Gly Ala Lys Pro Met Ile Glu Ala   155
ATA TTC CAA CCG GCA GAG GAG GAT GGA GGA TTA GGA GGA GCA AAA CCA ATG ATT GAG GCT   474

Gly Val Met Asn Gly Val Asp Tyr Val Phe Gly Ile His Ile Ser Ser Ser Tyr Pro Ser   175
GGA GTT ATG AAC GGT GTA GAT TAT GTA TTT GGA ATA CAT ATA TCG AGT AGT TAT CCT TCT   534

Gly Val Phe Ala Thr Arg Lys Gly Pro Ile Met Ala Thr Pro Asp Ala Phe Lys Ile Ile   195
GGA GTT TTC GCA ACT AGA AAA GGC CCT ATA ATG GCT ACG CCG GAC GCA TTC AAG ATA ATC   594

Val His Gly Lys Gly Gly His Gly Ser Ala Pro His Glu Thr Ile Asp Pro Ile Phe Ile   215
GTT CAC GGG AAG GGC GGT CAT GGT TCT GCT CCT CAT GAG ACT ATT GAC CCA ATT TTT ATA   654

Ser Leu Gln Ile Ala Asn Ala Ile Tyr Gly Ile Thr Ala Arg Gln Ile Asp Pro Val Gln   235
TCC TTA CAA ATA GCT AAC GCA ATC TAC GGC ATA ACA GCA AGG CAA ATT GAT CCA GTT CAA   714

Pro Phe Ile Ile Ser Ile Thr Thr Ile His Ser Gly Thr Lys Asp Asn Ile Ile Pro Asp   255
CCC TTT ATC ATA TCC ATT ACT ACA ATA CAT TCA GGT ACA AAG GAT AAC ATA ATA CCA GAT   774

Asp Ala Glu Met Gln Gly Thr Ile Arg Ser Leu Asp Glu Asn Val Arg Ser Lys Ala Lys   275
GAT GCC GAA ATG CAG GGA ACA ATT AGA AGT TTA GAC GAG AAC GTT AGA AGT AAG GCT AAG   834

Asp Tyr Met Arg Arg Ile Val Ser Ser Ile Cys Gly Ile Tyr Gly Ala Thr Cys Glu Val   295
GAC TAT ATG AGA AGA ATA GTT TCG TCA ATA TGT GGA ATC TAT GGT GCA ACT TGT GAG GTT   894

Lys Phe Met Glu Asp Val Tyr Pro Thr Thr Val Asn Asn Pro Glu Val Thr Asp Glu Val   315
AAA TTC ATG GAA GAC GTC TAT CCA ACT ACC GTA AAT AAC CCT GAG GTA ACT GAT GAG GTA   954

Met Lys Ile Leu Ser Ser Ile Ser Thr Val Val Glu Thr Glu Pro Val Leu Gly Ala Glu   335
ATG AAA ATT CTA TCT TCA ATA TCA ACA GTT GTT GAG ACA GAG CCA GTG CTA GGA GCG GAG  1014

Asp Phe Ser Arg Phe Leu Gln Lys Ala Pro Gly Thr Tyr Phe Phe Leu Gly Thr Arg Asn   355
GAC TTC TCC AGA TTC TTA CAG AAG GCT CCA GGA ACG TAT TTC TTT CTG GGA ACC AGA AAC  1074

Glu Lys Lys Gly Cys Ile Tyr Pro Asn His Ser Ser Lys Phe Cys Val Asp Glu Asp Val   375
GAA AAG AAA GGA TGC ATA TAT CCC AAT CAC AGC TCT AAG TTC TGT GTA GAT GAG GAC GTG  1134

Leu Lys Leu Gly Ala Leu Ala His Ala Leu Leu Ala Val Lys Phe Ser Asn Lys TER      395
CTA AAA TTA GGT GCC TTA GCT CAC GCA TTA TTG GCA GTA AAG TTC AGT AAT AAA TAA AGG  1194

GCT TAA TGC TAA ATG AGT ATT GGA AAT GGA GAA GGA AAA GGT TCT CAA TAT TTT GAG GAA  1254
TTC                                                                             1257
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FIG. 2. Nucleotide and deduced amino acid sequences of *cpsA*. The in-frame ATG and encoded Met are shown in boldface. The experimentally determined N-terminal sequence is underlined; nonmatching residues are italicized. The nucleotide sequence is numbered in the 5′-to-3′ direction beginning with the first *S. solfataricus* nucleotide sequenced.

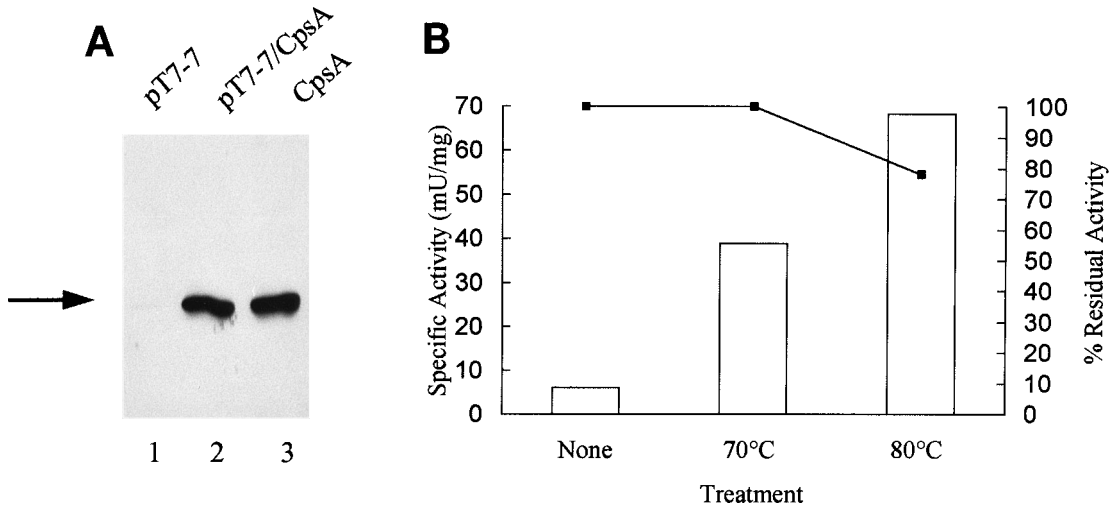

FIG. 3. Expression of *S. solfataricus* carboxypeptidase in *E. coli*. (A) Immunoblot analysis of *E. coli* BL21(DE3)[pLysE] transformed with plasmid pT7-7 (lane 1) or plasmid pT7-7/CpsA (lane 2). Lane 3, purified *S. solfataricus* carboxypeptidase (250 ng). (B) Specific (bars) and residual (squares) activities of carboxypeptidase after different heat treatments of a BL21(DE3)[pLysE, pT7-7/CpsA] crude extract in 50 mM Tris-HCl (pH 7.4)–5 mM 2-mercaptoethanol.

tests, extracts from BL21(DE3)[pLysE, pT7-7/CP14A] were heated for 3 min at either 70 or 80°C, and denatured proteins were eliminated by centrifugation. Total proteins were assayed with the Pierce reagent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (14). Immunoblotting was performed by standard techniques. Antibodies were raised in rabbits according to standard protocols (12). Specific anticarboxypeptidase antibodies were affinity purified essentially as described previously (26) with carboxypeptidase immobilized on nitrocellulose.

Enzymes, radioactive biochemicals, and synthetic oligonucleotides. Restriction enzymes were purchased from Promega (Madison, Wis.) and Boehringer Mannheim (Mannheim, Federal Republic of Germany). Radioactive biochemicals were obtained from Amersham (Amersham, United Kingdom). Oligonucleotides were obtained from Primm s.r.l. (Milan, Italy).

Bacteria strains, genomic library, and cloning vectors. *S. solfataricus* MT-4 (ATCC 49155) was kindly supplied by A. Gambacorta, Istituto per la Chimica di Molecole di Interesse Biologico del Consiglio Nazionale delle Ricerche, Arco Felice, Naples, Italy. *S. solfataricus* cells were grown aerobically in a minimal medium containing 2 g of yeast extract per liter as a carbon source and were collected in stationary phase as described previously (7). The expression library constructed in the λgt11 vector from *S. solfataricus* genomic DNA was kindly provided by M. Rossi, University of Napoli, Naples, Italy. *E. coli* Y1090 (Δ*lac*U169 *proA*$^+$ Δ*lon araD139 strA supF trpC22*::Tn*10 mcrA*/pMC9) and Y1089 (Δ*lac*U169 *proA*$^+$ Δ*lon araD139 strA hflA150 chr*::Tn*10*/pMC9) were used for plating λgt11 phages and obtaining lysogens, respectively (31). Plasmid pGEM-3Z (Promega) was used for subcloning and DNA sequencing, while plasmid pT7-7 (1) was used as an expression vector. *E. coli* JM101 [F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/*supE thi* Δ(*lac-proAB*) (23)] and BL21(DE3) [pLysE] [*hsdS gal*(λ*Its857 ind1 fSam7 nin5 lacUV5-T7* gene 1) (28)], respectively, were used as hosts for these plasmids. The carboxypeptidase expression plasmid was constructed as follows. The CP14A fragment containing the *cpsA* gene was obtained by *Eco*RI digestion of plasmid pGEM-3Z/CP14A (see Results and Discussion). After filling of recessed 3′ termini, the CP14A fragment was inserted into *Sma*I-cut pT7-7, generating plasmid pT7-7/CP14A. The CP14A fragment contains the complete sequence of the gene encoding carboxypeptidase and about 50 bp downstream of the translational stop codon; the 5′ terminus of this fragment contains the translational start codon, so that translation starts at the plasmid ATG.

Isolation and characterization of phage clones. Standard recombinant DNA techniques were performed as described by Sambrook et al. (23). The library was screened with affinity-purified antibodies by an adaptation of the method of Young and Davis (31). The insert of the positive clone was analyzed by restriction mapping, subcloned, and sequenced by the chain termination method with the Pharmacia T7 sequencing kit as suggested by the manufacturer.

Computer analysis. The amino acid sequence of carboxypeptidase was compared with those of other proteins in the SwissProt protein sequence database by using the FASTA program (18) as implemented in the Genetics Computer Group package of programs. Homology analyses with proteins of the carboxypeptidase family were conducted with the Gibbs sampling algorithm (15) as implemented in MACAW 2 (25). The algorithm locates relatively short patterns that are shared by otherwise dissimilar sequences and that reflect structural and functional constraints that arise from the energetic interaction among residues or between residue and ligand, irrespective of evolutionary history.

Nucleotide sequence accession number. The *cpsA* sequence shown in Fig. 2 has been deposited in the EMBL data library under accession number Z48497.

RESULTS AND DISCUSSION

Molecular cloning and nucleotide sequencing of *cpsA*, a carboxypeptidase-encoding gene from *S. solfataricus*. A carboxypeptidase-encoding gene was cloned by using an expression library constructed from *S. solfataricus* genomic DNA in the λgt11 vector. Phage clones were screened with affinity-purified anticarboxypeptidase antibodies; a positive phage, named λCP14, was isolated by repeated plaque purification. A lysogen for the positive phage was obtained by using *E. coli* Y1089 as the host, and the proteins expressed were analyzed by immunoblotting with anticarboxypeptidase (Fig. 1B) and anti-β-galactosidase (Fig. 1A) antibodies; an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible fusion protein of ca. 158 kDa was recognized by both antibodies (Fig. 1, lanes 2 and 4).

Antibodies affinity purified from the protein expressed by the λCP14A recombinant lysogen recognized a protein comigrating with carboxypeptidase in an *S. solfataricus* crude extract (Fig. 1C, lane 7). Moreover, in crude extracts of *E. coli* Y1089/λCP14, the carboxypeptidase specific activity was fourfold higher than it was in the absence of the inducer or in the control strain Y1089/λgt11 (data not shown). These findings suggest that the isolated *S. solfataricus* DNA insert in phage

TABLE 1. Substrate specificities of recombinant and native CpsA[a]

Substrate	Rate of hydrolysis (%)[b] with:	
	Recombinant CpsA	Native CpsA
CbzArg	100	100
CbzAsp	60	53
CbzGlyGlyPhe	196	237
CbzPhe	11	25
CbzAla	3	1

[a] Rates of hydrolysis were determined with the indicated benzyloxycarbonyl (Cbz) substrates as indicated in Materials and Methods. The equivalent of 100 ng of pure enzyme was used in each test.

[b] All values are percentages expressed relative to the rate of hydrolysis of CbzArg (100%) for each protein.

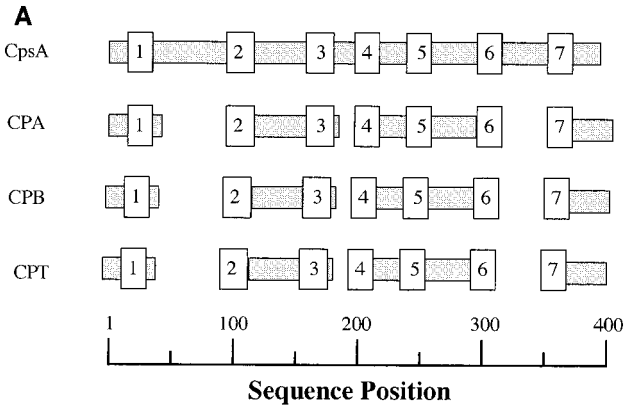

FIG. 4. Multiple alignment of carboxypeptidase from *S. solfataricus* (CpsA), mammalian carboxypeptidases A (CPA) and B (CPB), and carboxypeptidase from *T. vulgaris* (CPT). Proteins were aligned by using the Gibbs algorithm as implemented in MACAW with the BLOSUM62 substitution matrix. (A) Scheme highlighting the positions of the homology blocks within each protein. (B) Close-up view of homology blocks. Residues involved in zinc chelation are shown in boldface. Residues implicated in catalysis and conserved in CpsA are italicized. Secondary-structure assignments (2^{ary} Str) from the published three-dimensional structure of carboxypeptidase A are shown (α, α helix; β, β-strand; t, turn).

B

Block 1 **Block 2**

CpsA	16	DWIIQIRRKIHEYPELSYKEY	36 ...	93	LEFKSKVKGVM**H**ACG**HD**THVAMLL	116
CPA	33	VSKLQIGRSYEGRPIYVLKFS	53 ...	58	NRPAIWIDLGI**H**SR**E**WITQATGVW	81
CPB	30	VTQSVIGTTFEGRNMYVLKIG	50 ...	55	NKPAIFIDCGF**H**AR**E**WISPAFCQW	78
CPT	30	VKKFSIGKSYEGRELWAVKIS	50 ...	58	NEPEVLYTALH**H**AR**E**HLTVEMALY	81
2^{ary}	Str	ßßßßßßßß tt ßßßßßßßß			ßßßßßßß tt αααααααααα	

Block 3 **Block 4**

CpsA	162	DYVFGIHISSSYPSGVFATRKG	183 ...	197	HGKGGHGSAPHETIDP	212
CPA	126	WRKTRSVTSSSLCVGVDANRNW	147 ...	152	GKAGASSSPCSETYHG	167
CPB	123	WRKTRSTMAGSSCLGVRPNRNF	144 ...	149	CEVGASRSPCSETYCG	164
CPT	128	WRKNRQPNSGSSYVGTDLNRNY	149 ...	155	CCGGSSGSPSSETYRG	170
2^{ary}	Str	ααα			ß ß tt ttß	

Block 5 **Block 6**

CpsA	238	IISITTI**H**SGTKDNIIPDDA	257 ...	297	FMEDV*Y*PTTVNNP	309
CPA	189	FKAFLSI**H**SYSQLLLYPYGY	208 ...	243	IITTI*Y*QASGGSI	255
CPB	187	IKAYLTI**H**SYSQMMLYPYSY	206 ...	241	GATTI*Y*PAAGGSD	253
CPT	197	IKTLITF**H**TYSELILYPYGY	216 ...	250	QASDL*Y*ITDGDMT	262
2^{ary}	Str	ßßßßßßßß ßßßß			ßßßßt αα	

Block 7

CpsA	359	GCIYPNHSSKFCVD*E*	373
CPA	256	DWSYNQGIKYSFTF*E*	270
CPB	254	DWSYDQGIKYSFTF*E*	268
CPT	263	DWAYGQHKIFAFTF*E*	277
2^{ary}	Str	ααααατt ßßßßßß	

λCP14 encodes a carboxypeptidase. After removal of the phage arms by *Kpn*I-*Sac*I digestion, the λCP14 *Kpn*I-*Sac*I fragment was subcloned in pGEM-3Z, generating plasmid pGEM-3Z/λCP14. The λCP14 insert was then cleaved into three fragments that were subcloned in pGEM-3Z, generating plasmid pGEM-3Z/CP14A, pGEM-3Z/CP14B, and pGEM-3Z/CP14C. The complete sequence of the CP14A fragment was determined with synthetic oligonucleotides as primers. Within fragment CP14A, an 1,179-bp open reading frame encoding a protein of 393 amino acids was found (Fig. 2). The predicted protein would have a molecular mass of 43,068 Da, in good agreement with the enzyme molecular mass assessed by SDS-PAGE (42 kDa per subunit). One ATG codon (in boldface in Fig. 2) at position 10 was in frame with λgt11 *lacZ*. The amino acid sequence encoded between nucleotides 10 and 102 (underlined in Fig. 2) matched the experimentally determined N-terminal amino acid sequence of pure *S. solfataricus* carboxypeptidase, with four exceptions (shown in italics in Fig. 2). This finding indicates that translation starts at position 10 and confirms the identity of the cloned gene. The observed discrepancy might be due to mistakes in the amino acid sequence determination, to strain variation, or to the presence of different carboxypeptidase isoforms. We propose to call this gene *cpsA*.

As reported for other *S. solfataricus* protein-encoding genes (4–6, 19–21, 24), the codon usage of the *cpsA* gene shows a strong preference towards A and T, reflecting the low G+C content of the DNA from this organism. The overall A+T content of the *cpsA* gene is 60%, rising to 66% when only the third position is considered, with Trp and Met excluded. Only AGG and AGA codons, which are very rare in *E. coli* but common in eukaryotes, are used for arginine, in agreement with data previously reported (4–6).

No putative terminator signal matching the consensus (TTT TTYY) proposed by Reiter et al. (22) was found downstream of the termination codon.

Expression of *S. solfataricus* carboxypeptidase in *E. coli*. *E. coli* carboxypeptidase expression plasmids were constructed as described in Materials and Methods and transformed into strain BL21(DE3)[pLysE]. Carboxypeptidase activity was as-

sayed in *E. coli* BL21(DE3)[pLysE] transformed with plasmid pT7-7/CP14A, encoding carboxypeptidase, and the control plasmid pT7-7. A 42-kDa protein was specifically detected by anticarboxypeptidase immunopurified antibodies (Fig. 3A) in BL21(DE3)[pLysE, pT7-7/CP14A] and not in BL21(DE3)[pLysE, pT7-7]. Accordingly, carboxypeptidase activity was detected in BL21(DE3)[pLysE, pT7-7/CP14A] but not in BL21(DE3)[pLysE, pT7-7]. Assuming that the specific activity of the expressed enzyme is the same as that of the carboxypeptidase from *S. solfataricus*, we calculated that under these conditions active carboxypeptidase accumulated to about 0.3% of total *E. coli* proteins.

In order to better characterize the activity of the recombinant protein, thermal stability and substrate specificity were tested. Under the assay conditions used, no activity was detectable in crude extracts of the control strain BL21(DE3)[pLysE, pT7-7] (data not shown). Figure 3B shows that after 3 min at 70 and 80°C, 100 and 80%, respectively, of the initial activity was retained, with over a 10-fold increase in specific activity. The *Sulfolobus*-produced enzyme has been shown to have a similar thermal stability (reference 30 and data not shown).

E. coli-expressed CpsA showed a broad substrate specificity, as shown by using an enzyme which was partially purified (70% purity) according to a modification (29a) of a published procedure (3). A substrate specificity comparable to that of the purified *S. solfataricus* enzyme was displayed, as demonstrated by the release of basic, aromatic, and acid amino acids from benzyloxycarbonyl-amino acids (Table 1 and reference 3).

Sequence homology. Comparison of the amino acid sequence of the *S. solfataricus* carboxypeptidase with those of proteins in the SwissProt data bank did not show significant similarities to carboxypeptidases. Global alignment programs like FASTA often lack the sensitivity necessary to detect low-level, local homologies. We thus used a recently described algorithm to test for local homologies between the protein encoded by the cloned *S. solfataricus* gene and other carboxypeptidases, namely, mammalian carboxypeptidases A and B and *T. vulgaris* carboxypeptidase T. This analysis identified seven major homology blocks, which are schematically shown as white boxes in Fig. 4A.

Biochemical and genetic analyses have identified several key residues in metallocarboxypeptidases (see reference 2 for a comprehensive review). In bovine carboxypeptidase A, His-69, Glu-72, and His-196 are involved in zinc chelation. Figure 4B shows that two histidines (His-104 and His-245) are aligned to His-69 and His-196, respectively, of the bovine enzyme. No equivalent of Glu-72 is found; however, Asp-109 might be a viable substitute. Residues involved in catalysis in carboxypeptidase A include Arg-127, Asn-144, Arg-145, Tyr-248, and Glu-270. Homologous residues for the last three have been identified at positions 181, 302, and 373, respectively.

The absence of Arg and Asn at positions 127 and 144 (carboxypeptidase A numbering) is intriguing. Both Arg-127 and Asn-144 have been proposed to act as hydrogen bond donors; the former would act in stabilizing the oxyanion in the transition state, and the latter would cooperate with Arg-145 and Tyr-248 in hydrogen bonding the C-terminal carboxylate of the substrate. Arg-127 has also been proposed to act as the nonmetal location of the intact carbonyl just prior to catalysis (reviewed in reference 2). Despite the wealth of structural information available on carboxypeptidase A, few mutagenesis experiments have been designed to directly probe the roles of residues thought to be involved in catalysis and binding. The first of these experiments addressed the role of Tyr-248, which, on the basis of structural and chemical modification experiments, was suggested to be the general acid catalyst required for peptide bond hydrolysis. The experiments showed that the phenolic hydoxyl of Tyr-248 was not essential for catalysis, although it was participating in substrate binding, since the mutated enzyme had the same k_{cat} as the wild-type enzyme had but a higher K_m for some, but not all, substrates (11). Tyr-163 and Thr-180 are found aligned with Arg-127 and Asn-144 and might functionally replace these residues. Site-directed mutagenesis and structural studies are under way in order to define the roles of the above-mentioned residues in the CpsA catalytic mechanism and to clarify the molecular basis of the unusually broad substrate specificity and chemio- and thermostability of this interesting enzyme.

ACKNOWLEDGMENTS

We thank M. Rossi for the generous gift of the *S. solfataricus* gene bank and A. Gambacorta for supplying the MT-4 strain of *S. solfataricus.*

This work has been supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (40% program) and the Consiglio Nazionale delle Ricerche.

REFERENCES

1. **Bianchi, M. E.** 1991. Production of functional rat HMG1 protein in *Escherichia coli*. Gene **104:**271–275.
2. **Christianson, D. W., and W. N. Lipscomb.** 1989. Carboxypeptidase A. Acc. Chem. Res. **22:**62–69.
3. **Colombo, S., S. D'Auria, P. Fusi, L. Zecca, C. A. Raia, and P. Tortora.** 1992. Purification and characterization of a thermostable carboxypeptidase from the extreme thermophylic archaebacterium *Sulfolobus solfataricus*. Eur. J. Biochem. **206:**349–357.
4. **Colombo, S., M. Grisa, P. Tortora, and M. Vanoni.** 1994. Molecular cloning, nucleotide sequence and expression of a *Sulfolobus solfataricus* gene encoding a class II fumarase. FEBS Lett. **337:**93–98.
5. **Cubellis, M. V., C. Rozzo, P. Montecucchi, and M. Rossi.** 1990. Isolation and sequencing of a new β-galactosidase-encoding archaebacterial gene. Gene **94:**89–94.
6. **Cubellis, M. V., C. Rozzo, G. Nitti, M. I. Arnone, G. Marino, and G. Sannia.** 1989. Cloning and sequencing of the gene coding for aspartate aminotransferase from the thermoacidophilic archaebacterium *Sulfolobus solfataricus*. Eur. J. Biochem. **186:**375–381.
7. **De Rosa, M., A. Gambacorta, B. Nicolaus, P. Giardina, E. Poerio, and V. Buonocore.** 1984. Glucose metabolism in the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus*. Biochem. J. **224:**407–414.
8. **Doi, E., D. Shibata, and T. Matoba.** 1981. Modified colorimetric ninhydrin methods for peptidase assays. Anal. Biochem. **118:**173–184.
9. **Folk, J. E.** 1971. Carboxypeptidase B, p. 57–79. *In* P. D. Boyer (ed.), The enzymes, 3rd ed. vol. 3. Academic Press, New York.
10. **Fricker, L. D., and S. H. Snyder.** 1982. Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. Proc. Natl. Acad. Sci. USA **79:** 3886–3890.
11. **Gardell, S. J., C. S. Craick, D. Hilvert, M. S. Urdea, and W. Rutter.** 1985. Site-directed mutagenesis shows that tyrosine 248 of carboxypeptidase A does not play a crucial role in catalysis. Nature (London) **317:**551–555.
12. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Hartsuck, J. A., and W. N. Lipscomb.** 1971. Carboxypeptidase A, p. 1–56. *In* P. D. Boyer (ed.), The enzymes, 3rd ed. vol. 3. Academic Press, New York.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Lawrence, C. E., S. F. Altschul, M. S. Boguski, J. S. Liu, A. F. Neuwald, and J. C. Wotton.** 1993. Detecting subtle sequence signals: a Gibbs sampling strategy for multiple alignment. Science **262:**208–214.
16. **Lee, S.-H., E. Minagawa, H. Taguchi, H. Matsuzawa, T. Ohta, S. Kaminogawa, and K. Yamauchi.** 1992. Purification and characterization of a thermostable carboxypeptidase (carboxypeptidase *Taq*) from *Thermus aquaticus* YT-1. Biosci. Biotechnol. Biochem. **56:**1839–1844.
17. **Levin, Y., R. A. Skidgel, and E. G. Erdös.** 1982. Isolation and characterization of the subunits of human plasma carboxypeptidase N (kininase I). Proc. Natl. Acad. Sci. USA **79:**4618–4622.
18. **Lipman, W. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1441.
19. **Olsen, G. J., N. R. Pace, M. Nuell, B. P. Kaine, R. Gupta, and C. R. Woese.** 1985. Sequence of 16srRNA gene from the thermoacidophilic archaebacterium *Sulfolobus solfataricus* and its evolutionary implications. J. Mol. Evol. **22:**301–307.
20. **Pisani, F. M., C. De Martino, and M. Rossi.** 1992. A DNA polymerase from

the archaeon *Sulfolobus solfataricus* shows sequence similarity to family B DNA polymerases. Nucleic Acids Res. **20:**2711–2716.
21. **Ramirez, C., and A. T. Matheson.** 1991. A gene in the archaebacterium *Sulfolobus solfataricus* that codes for a protein equivalent to the alpha subunits of the signal recognition particle receptor in eukaryotes. Mol. Microbiol. **5:**1687–1693.
22. **Reiter, W. D., P. Palm, A. Henschen, F. Lottspeich, W. Zillig, and B. Grampp.** 1987. Identification and characterization of the genes encoding three structural proteins of the *Sulfolobus* virus-like particle SSV1. Mol. Gen. Genet. **206:**144–153.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sanangelantoni, A. M., D. Barbarini, G. Di Pasquale, P. Cammarano, and O. Tiboni.** 1990. Cloning and nucleotide sequence of an archaebacterial glutamine synthetase gene: phylogenetic implications. Mol. Gen. Genet. **221:**187–194.
25. **Schuler, G. D., S. F. Altschul, and D. J. Lipman.** 1991. A workbench for multiple alignment and analysis. Proteins **9:**180–190.
26. **Smith, D. E., and P. A. Fisher.** 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polipeptides immobilized on nitrocellulose blots. J. Cell Biol. **99:**20–28.
27. **Smulevitch, S. V., A. L. Osterman, O. V. Galperina, M. V. Matz, O. P. Zagnitko, R. M. Kadyrov, I. A. Tsaplina, N. V. Grishin, G. G. Chestukhina, and V. M. Stepanov.** 1991. Molecular cloning and primary structure of *Thermoactinomyces vulgaris* carboxypeptidase T. A metalloenzyme endowed with dual substrate specificity. FEBS. Lett. **291:**75–78.
28. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
29. **Teplyakov, A., K. Polyakov, G. Obmolova, B. Strokopyton, I. Kuranova, A. Osterman, N. Grishin, S. Smulevitch, O. Zagnitko, O. Galperina, M. Matz, and V. M. Stepanov.** 1992. Crystal structure of carboxypeptidase T from *Thermoactinomyces vulgaris*. Eur. J. Biochem. **208:**201–288.
29a. **Tortora, P.** Unpublished work.
30. **Villa, A., L. Zecca, P. Fusi, S. Colombo, G. Tedeschi, and P. Tortora.** 1993. Structural features responsible for kinetic thermal stability of a carboxypeptidase from the archaebacterium *Sulfolobus solfataricus*. Biochem. J. **295:** 827–831.
31. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA **80:**1194–1198.