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# "Protease I" of *Escherichia coli* Functions as a Thioesterase In Vivo

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***Escherichia coli* protease I is assayed as an esterase active with certain synthetic model chymotrypsin substrates. However, the gene encoding protease I has the same DNA sequence and genomic location as *tesA*, a gene that encodes *E. coli* thioesterase I. We report that both hydrolase activities utilize the same active site and demonstrate that the protein functions as a thioesterase in vivo.**

*Escherichia coli* protease I was first reported by Pacaud and Uriel (13). The partially purified enzyme was reported to convert the native form of polynucleotide phosphorylase into a smaller, active form of the enzyme. Later, Pacaud et al. (12) further purified protease I by using *N*-acetyl-DL-phenylalanine-2-naphthyl ester (NAPNE) as a model substrate and reported cleavage of oxidized bovine insulin by the purified enzyme. Kowit et al. (7) reported the isolation of an *E. coli* mutant deficient in NAPNE hydrolysis and also reported that purified protease I failed to hydrolyze casein. *Salmonella typhimurium* mutants unable to cleave NAPNE were isolated by Miller et al. (9), who mapped the gene and designated it *apeA*. Recently, Ichihara et al. (5) screened the Clarke-Carbon clone bank for strains that overproduced an esterase activity that hydrolyzed another synthetic protease I substrate, *N*-benzyloxycarbonyl-L-phenylalanine β-naphthyl ester. One class of these clones was shown to encode protease I, and the gene was subcloned, sequenced, and designated *apeA* by analogy with the *S. typhimurium* mutant gene. They also purified the enzyme to homogeneity and showed that its properties were those reported by Pacaud and coworkers (12, 13).

In 1967, Kass and coworkers reported the presence of thioesterase activity in extracts of *E. coli* (6). Two distinct enzymes that catalyze the hydrolysis of fatty acyl coenzyme A (acyl-CoA) substrates were detected after fractionation of cell extracts (1). Barnes and Wakil partially purified and characterized the lower-molecular-weight enzyme, thioesterase I (2). Recently, we reported the cloning and DNA sequencing of the *tesA* gene encoding *E. coli* thioesterase I as well as studies of the physiological role of the enzyme (4).

A comparison of our data (4) and those of Ichihara et al. (5) showed that the *apeA* and *tesA* genes have identical nucleotide sequences and the same location on the *E. coli* chromosome. Moreover, the proteins purified by following either enzyme activity are both of periplasmic origin and have identical molecular weights and amino-terminal sequences (4, 5). Thus, protease I and thioesterase I are the same protein, which raises the question of which activity (if not both or neither) is physiologically relevant. We report that the same active site is used for both fatty acid and amino acid substrates and show that overproduction of this protein results in accumulation of free fatty acids, indicating that the protein has thioesterase activity in vivo.

First, we performed substrate competition assays with the purified protein to test whether the amino acid and fatty acid substrates used a common active site. Because of the insolubility of the amino acid substrates under the assay conditions used for thioesterase activity, we assayed the effect of palmitoyl-CoA on esterase ("protease I") activity by using NAPNE (Fig. 1A) or *N*-benzoyloxycarbonyl-L-tyrosine-*p*-nitrophenyl ester (NbzYNPE) (Fig. 1B) as the substrate. The assays were done as described by Pacaud et al. (12). In the presence of 0.1 mM palmitoyl-CoA, we observed a complete inhibition of NAPNE hydrolysis (Fig. 1). However, palmitoyl-CoA is known to have detergent properties which could result in denaturation of the protein (2). Therefore, we used 0.1 mM decanoyl-CoA, another detergent-like thioester that is not a substrate for thioesterase I (1, 4) as a control and found that protease activity was not inhibited by this compound (Fig. 1A). The rates of hydrolysis were 21.3 μmol/min/mg for palmitoyl-CoA and 40.3 μmol/min/mg for NAPNE. The limited solubility of NAPNE precluded a kinetic analysis of the inhibition of the hydrolysis by palmitoyl-CoA and thus we turned to a more soluble protease I substrate, NbzYNPE (13).

The hydrolysis of NbzYNPE was competitively inhibited by palmitoyl-CoA (Fig. 1B). However, at higher NbzYNPE concentrations, the plots indicated a mixed (competitive plus noncompetitive) inhibition. The noncompetitive inhibition is probably due to the inhibition of the enzyme activity observed at high substrate concentrations (data not shown). We were unable to assay inhibition of palmitoyl-CoA hydrolysis by NbzYNPE because of the poor solubility of NbzYNPE and the spectral overlap of NbzYNPE with the chromatophore used to assay thioesterase activity.

These results indicate that hydrolysis of both fatty acid and amino acid substrates involves the same active site. This result is strongly supported by prior findings that the hydrolysis of both model protease (13) and acyl-CoA substrates (2) is inhibited by the active serine inhibitor diisopropyl fluorophosphate, which we showed to react specifically with a single serine residue (4).

Is the product of the *tesA/apeA* gene a thioesterase, a protease, or both? From the in vitro results of Barnes and Wakil (2) and Spencer et al. (15), expression of thioesterase I in the *E. coli* cytosol should give rise to intracellular free fatty acids (FFA) by cleavage of the thioester bonds of the acyl-acyl carrier protein (acyl-ACP) intermediates of fatty acid and complex lipid (phospholipid and lipid A) synthesis. However, these intermediates are cytosolic, whereas TesA/ApeA is a periplasmic protein (4, 5), and thus compartmentalization should preclude FFA production. However, we had noted (as had Ichihara et al. [5]) that upon overproduction of the

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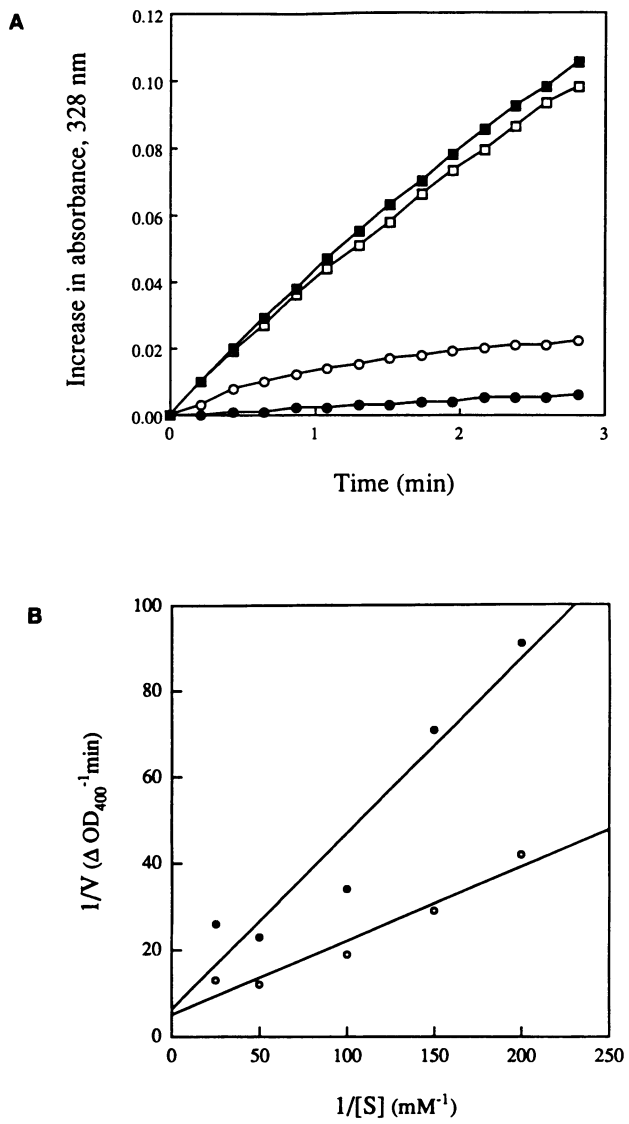


FIG. 1. Competition between substrates. (A) A freshly prepared solution of 0.3 mM NAPNE in 50 mM potassium phosphate buffer (pH 7.5) containing 20% dioxane was placed in the cuvette, and the enzyme reaction was started by adding 0.6 μg of thioesterase I (purified to near homogeneity [4]). Activity was monitored by the increase in $A_{328}$. Neither fatty acid nor CoA inhibited the reaction. Symbols: ●, no enzyme; □, plus thioesterase I; ○, plus thioesterase I and palmitoyl-CoA (0.1 mM final concentration); ■, plus thioesterase I and decanoyl-CoA (0.1 mM final concentration). (B) Lineweaver-Burk plot of competitive inhibition between substrates. Protease activity was measured with NbzYNPE in the absence and presence of palmitoyl-CoA (5 μM final concentration) as an inhibitor. Assays were done at pH 7.5 at room temperature as described by Pacaud et al. (12). Various amounts of freshly prepared 2 mM solution of the substrate made in dioxane were added to 100 mM potassium phosphate buffer (pH 7.5) just prior to the assay. The enzyme reaction was then started by adding 0.09 μg of thioesterase I (purified to near homogeneity [4]). Activity was monitored by the increase in $A_{400}$ against that in a control cuvette without the enzyme. The velocities were expressed as changes in optical density per minute at 400 nm. Symbols: ○, no inhibitor; ●, palmitoyl-CoA (5 μM final concentration). Plotting was done by linear regression with Sigma Plot 4.11.

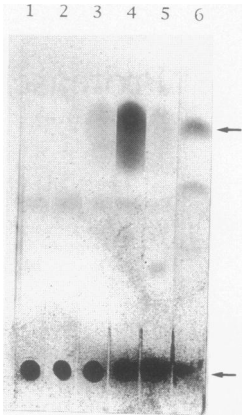


FIG. 2. Thin-layer chromatographic analysis of [1-$^{14}$C]acetate-labeled lipids. Two milliliters of cell cultures grown in rich broth (4) overnight were labeled with [1-$^{14}$C]acetate (10 μCi/ml) at 37°C for 5 min. Cells were then washed twice, and lipids were extracted by the Bligh and Dyer method (3). The samples were dried under $N_2$ gas, resuspended in $CH_3OH$-$CH_3Cl$ (2:1, vol/vol), and applied to a silica gel G thin-layer plate. The amount loaded in each lane was normalized by cell density. The plate was developed in petroleum ether-ether-acetic acid (70:30:2, by volume) and autoradiographed. All strains are derivatives of strain HC71 (a *fadE62* derivative of the K-12 strain LE392) and were constructed by P1 transduction. Lane 1, Tes$^+$ strain HC71; lane 2, Δ*tesA*::Kan$^r$ Δ*tesB*::Cml$^r$ strain HC74; lane 3, Tes$^+$ strain HC71 carrying pHC64, a Δ*tesA* derivative of the *tesA* plasmid pHC63 (4); lane 4, Tes$^+$ strain HC71 carrying the *tesA* plasmid pHC63 (4); lane 5, Tes$^+$ strain HC71 carrying the *tesB* plasmid pUC120*tesB* (10); lane 6, [9,10-$^3$H]oleic acid. The upper arrow indicates the FFA fraction, and the lower arrow indicates the phospholipid fraction.

TesA/ApeA protein, a portion of the enzyme activity remained in the cytosolic fraction. This result is presumably due to titration of a limiting cellular component required for export to the periplasm (e.g., a chaperonin). Indeed, we found that strains that overproduced TesA/ApeA accumulated FFA (Fig. 2), whereas wild-type strains and thioesterase-defective mutants produced no detectable FFA. We also found that TesA/ApeA-overproducing strains accumulated intracellular FFA when incubated with exogenous [9,10-$^3$H]oleic acid (data not shown).

These data indicated that TesA/ApeA acted as a thioesterase in vivo. The FFA produced in the absence of exogenous fatty acids is attributed to cleavage of acyl-ACP molecules, whereas the FFA observed when cells are supplemented with exogenous fatty acids is attributed to cleavage of the acyl-CoA intermediates of the β-oxidation (*fad*) pathway. Exogenously added long-chain FFA do not efficiently enter the *E. coli* cytosol as FFA but do so as acyl-CoA esters, since transport is coupled to acyl-CoA synthetase action (11). Thus, the FFA that we observed can be attributed to cleavage of these CoA esters by TesA/ApeA present in the cell cytosol. Recently, we constructed a mutant *tesA* gene with a precise deletion of the leader (signal sequence). Preliminary results indicate that strains expressing this leaderless TesA protein accumulate extremely high levels of FFA by hydrolysis of endogenous substrates (unpublished data).

It should be noted that phospholipids and other acylated molecules are also possible sources of FFA. However, the TesA/ApeA protein fails to cleave nonactivated oxygen esters (the ester bonds of the model chymotrypsin substrates are unusually labile because of the attached chromatophores) (12, 13). Moreover, although the phospholipids and other acylated

molecules of *E. coli* are all membrane bound and thus accessible to the periplasm (the normal location of TesA/ApeA), no FFA are produced in wild-type strains (Fig. 2).

In vitro, the TesA protein efficiently hydrolyzes two dissimilar types of molecules, activated oxygen esters (12, 13) and both acyl-CoA (2) and acyl-ACP (15) thioesters, whereas short-chain thioesters (e.g., decanoyl-CoA) are not hydrolyzed. A clue to this unusual specificity is that only the activated esters of nonpolar amino acids are hydrolyzed (12), and thus hydrophobicity and a readily hydrolyzed ester (or thioester) bond seem to be the determinants of substrate activity. It may be that binding to the enzyme requires a critical hydrophobic surface area and that short-chain acyl-CoAs and activated esters of hydrophilic amino acids fall below this minimum. A precedent for this type of specificity is mammalian acyl-CoA binding protein, which displays high binding affinity only toward long-chain ($\geq C_{14}$) acyl-CoA molecules (14). The X-ray structure of the complex of the protein with palmitoyl-CoA shows that only long-chain acyl-CoA molecules, not short-chain acyl-CoA molecules, can fill the hydrophobic binding site and allow the cooperative interactions required for tight binding (8).

Several laboratories have studied the TesA/ApeA enzyme, and only one reported hydrolysis of proteins by protease I (rather than synthetic model substrates). First, Pacaud and Uriel reported that partially purified protease I cleaved *E. coli* polynucleotide phosphorylase to a smaller form (13). However, the cleavage required 30 h. Pacaud et al. (12) then reported cleavage of denatured insulin with a more purified enzyme preparation, but the conditions were again extreme; a week at 37°C gave only partial hydrolysis, with 10 mol% of the total protein being protease I. Thus, the data of Pacaud and coworkers are readily explained by minor contamination of the enzyme preparations with a true protease. Consistent with this interpretation, Kowit et al. (7) were unable to detect hydrolysis of casein by a sample of the purified enzyme obtained from Pacaud. In addition, the purified protein lacks amidase activity (a characteristic of proteases) (13). Moreover, mutants lacking the enzyme grow normally on peptides and show no alterations in protein turnover rates (5, 7, 9).

For these reasons, we believe that TesA is not a protease and that the model protease substrates are acting as analogs of acyl-CoA rather than as polypeptide analogs. Moreover, as previously discussed, the spatial arrangement of the Ser and His elements of the TesA protein has a stronger resemblance to that found in known thioesterases than the arrangement of these elements in known proteases (4). From these data, we conclude that TesA does not have protease activity and that the designation protease I is a misnomer. Therefore, the *tesA* gene designation should replace *apeA*, since *tesA* reflects enzymatic hydrolysis of a natural substrate. The realization that protease I is instead a thioesterase underscores the problems involved in the use of synthetic esters as the sole criterion for defining a protease or peptidase (as previously pointed out by Kowit et al. [7]).

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