Generation of a Thermostable and Denaturant-Resistant Peptide Ligase[†]

Koman Joe,[‡,§] Thor J. Borgford,[‡,||] and Andrew J. Bennet*[,⊥]

Departments of Chemistry and of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, Canada V5A 1S6

Received February 18, 2004; Revised Manuscript Received April 2, 2004

ABSTRACT: The construction and characterization of a novel, thermostable, peptide ligase are described. Three amino acid substitutions were introduced into the secreted bacterial protease *Streptomyces griseus* protease B (SGPB). Mutations were chosen on the basis of two separate observations: (i) that a single substitution of the nucleophilic serine (S195A) created an enzyme with significant peptide-ligation activity, albeit greatly reduced stability [(2000) *Chem. Biol. 7*, 163], and (ii) that a pair of substitutions in the substrate-binding pocket (T213L and F228H) greatly increased the thermostability of the wild-type enzyme [(1996) *J. Mol. Biol. 257*, 233]. The triple mutant, named streptoligase, was found to catalyze peptide ligation (aminolysis of both a thiobenzyl ester and a *p*-nitroanilide-activated peptide) efficiently in nondenaturing and denaturing conditions including SDS (0.5% w/v) and guanidine hydrochloride (4.0 M). Moreover, streptoligase exhibited a half-live for unfolding of 16.3 min at 55 °C in the absence of stabilizing substrates. The fraction of the streptoligase-catalyzed reaction that gave coupled product with the acceptor peptide FAASR-NH_2 was greater for the *p*-nitroanilide donor (Sc-AAPF-*p*NA) than for the benzyl thioester substrate (Sc-AAPF-SBn). These observations are consistent with ligation proceeding through an acyl-enzyme intermediate involving histidine-57. In the case of the thioester donor the triple mutant promotes the direct attack of water on the thioester carbonyl carbon, in addition to hydrolysis occurring at the stage of the acyl-enzyme intermediate. The strategy of multiple point mutations outlined in this study may provide a general means of converting enzymes with chymotrypsin-like protein folds into peptide ligases.

Oligonucleotide-directed mutagenesis techniques make possible virtually any site-specific alterations to the primary sequence of proteins, and such changes have proved to be powerful methods for dissecting relationships between protein structure and function. Although powerful and useful, conventional molecular biology techniques are generally restricted to the use of 20 amino acids dictated by the genetic code. Indeed, a significantly greater range of protein structure and function modification is feasible if nonnatural amino acids are incorporated into the polypeptide chain. Solid-phase peptide synthesis permits routine incorporation of natural and nonnatural amino acids into short oligopeptides. Regrettably, these methods are costly and synthetically challenging, so that the routine chemical synthesis of entire proteins is not yet practical.

Other modern techniques for the incorporation of nonnatural amino acids into peptides include, but are not restricted to, (a) native chemical ligation (*1*), (b) expressed protein ligation (*2*), (c) extension of the genetic code (*3*), and (d) use of protease enzymes and active site mutants thereof (*4*).

Kent and co-workers developed nonenzymatic methods to ligate peptides in aqueous phases (*5*). Generally, these reactions involve a donor peptide having a carboxyl terminus that is a thioester and an acceptor peptide possessing an unblocked cysteine residue. Coupling proceeds through an intermolecular S → S transacylation reaction followed by a spontaneous intramolecular S → N acyl transfer, resulting in a peptide bond between the donor and acceptor peptides (*1*). Recently, this technique was extended to reactions such as the Staudinger ligation where a peptide bond is formed via the reaction of a thioester and an azide (*6*, *7*). Of note, the development of a method to generate recombinant proteins activated with carboxy-terminal thioesters (*8*, *9*) has increased the versatility and accessibility of chemical ligation (*2*, *10*).

Applying a much different strategy, Schultz and coworkers have extended the genetic code using nonnatural amino acid tRNA in order to incorporate novel amino acids such as L-3-(2-naphthyl)alanine (*11*) and *p*-azido-L-phenylalanine (*12*) into recombinant proteins. Other researchers are using various ribosomal-based strategies to incorporate novel amino acids into peptides (*13*−*16*). Although the genetic code has been expanded, this method is still restricted by the ability of the respective tRNA synthases to accept nonnatural amino acids. Also, several simultaneous nonnatural substitutions are not yet possible using this technology.

[†] This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC).

* Corresponding author: e-mail, bennet@sfu.ca; fax, (604) 291-3765; phone, (604) 291-3532.

[‡] Department of Molecular Biology and Biochemistry.

[§] Current address: Inex Pharmaceuticals, 8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8.

[||] Current address: Twinstrand Therapeutics, 8081 Lougheed Highway, Burnaby, BC, Canada V5A 1W9.

[⊥] Department of Chemistry.

10.1021/bi0496337 CCC: $27.50
Published on Web 05/15/2004

Lastly, as Bordusa pointed out in a recent review (*4*), many enzymatic peptide-coupling strategies are currently in use. Indeed, almost all of these ligating technologies are conducted in water using unprotected peptides where intermolecular peptide bond formation is thermodynamically unfavorable with respect to the predominant species in solution, namely, ammonium and carboxylate ions (*17*). As a consequence, activated substrate peptides are used, and the reaction conditions are tailored to allow "kinetic" trapping of the ligation products.

A notable advantage of enzyme-catalyzed ligation is the potential for coupling peptides without residual thiols (from thioesters) as is the case for native chemical ligation. Proteases or their variants are able to ensure that the coupling reaction proceeds with the desired regiospecificity and the site of reaction does not depend on leaving group chemistry. In theory, any junction can be created between two peptides without introducing a thiol into the nascent peptide.

Wells and co-workers reported the conversion of the protease subtilisin BPN′ into the peptide-ligating catalyst "subtiligase" (*18*) by mutating the enzyme's catalytic serine to a cysteine residue (S221C). The mutation produced an enzyme with a substantially reduced amidase activity, rendering the enzyme incapable of hydrolyzing ordinary peptide bonds (*18*, *19*). Despite the reduction in hydrolytic activity, the newly introduced cysteine of subtiligase was readily acylated by glycolate ester-activated peptides, and subsequent attack on the thioester intermediate by amine nucleophiles generated a ligated product. Improved variants of subtiligase possessing increased activities and oxidative stabilities have been selected using phage-display techniques (*20*). Wells and co-workers were also able to improve the stability of subtiligase by incorporating five additional mutations into subtiligase to produce "stabiligase", an enzyme that retained nearly 50% activity in 4 M guanidine hydrochloride (*19*).

A different approach to enzymatic ligation involving the chymotrypsin-like serine protease *Streptomyces griseus* protease B (SGPB)[1] was reported by Elliott et al. (*21*). In this case the active site nucleophile (Ser195) was substituted with a nonnucleophilic residue (alanine or glycine). The ensuing mutants were able to couple donor peptides with thioesters, esters, and anilide substrates (*21*). The authors proposed a two-step reaction mechanism wherein coupling occurs through a transiently acylated histidine that is deacylated by the terminal amine of an acceptor peptide. Coupling efficiencies were determined by the competing rates of aminolysis versus hydrolysis (*21*). A major drawback to the two first-generation SGPB-derived peptide-ligating catalysts was their greatly reduced thermostabilities (S195A is completely inactivated in 1 min at 40 °C) relative to the wild-type SGPB ($t_{1/2}$ = 8.8 min at 55 °C). Therefore, the objective of the current study was to stabilize the active site variants of SGPB through secondary mutations. Potentially stabilizing substitutions were suggested by an unrelated study of the enzyme SGPB.

During its natural biosynthesis, wild-type SGPB is made as a proprotein zymogen that must be proteolytically processed to generate mature protease. This critical processing event is autocatalytic; consequently, the amino acid at the junction between pro and mature regions of the zymogen is leucine, a good substrate for the enzyme (i.e., in addition to having a chymotrypsin-like protein fold, SGPB has chymotrypsin-like activity, recognizing large hydrophobic or aliphatic amino acid side chains). Sidhu et al. took advantage of this observation to develop a cell-based screening strategy for SGPB variants with altered primary specificity (*22*). The leucine at the promature junction of SGPB was mutated to residues that are not ideal substrates for wild-type enzyme (for example, Met and Val), and on their own, such substitutions either completely abolished or impaired the expression of active SGPB protein. However, expression of variant forms of SGPB could be rescued/restored via combinatorial substitutions within the substrate-binding pockets of the enzyme. The restoration of protease activity was observed by the appearance of zones of clearing on milk plates.

An unanticipated consequence of these library selection studies was the appearance of variants with significantly enhanced thermostabilities. In particular, the screen performed with Phe at the promature junction produced four distinct variants with increased zones of clearing in comparison to that furnished by the wild-type SGPB producing *Escherichia coli* (*22*). Although none of the variants exhibited increased k_{cat}/K_m values toward substrates that contained Phe at the P1 site, three of the variants (SGPBFrl, SGPBFr12, and SGPBFr13) had greatly increased thermostabilities at 55 °C when compared to SGPB (*22*). Specifically, the SGPB variant SGPBFr13 with two amino acid substitutions had a $t_{1/2}$ at 55 °C that was approximately 10 times longer than that of the wild-type SGPB. In an effort to improve the stability and hence peptide-ligating utility of the S195G and S195A variants, the secondary substitutions T213L and F228H originally found in SGPBFr13 were introduced into the active site of the SGPB S195A variant to give a triple mutant enzyme hereafter referred to as "streptoligase".

MATERIALS AND METHODS

Materials. All genetic manipulation steps were performed as previously outlined (*23*) in the *E. coli* strain DH5α. Genes of interest were expressed in *Bacillus subtilis* DB104, a protease-deficient strain. *N*-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sc-AAPF-*p*NA) was purchased from Sigma. *N*-Succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester (Sc-AAPF-SBn) was purchased from Bachem. Phe-Ala-Ala-Ser-Arg-NH_2 (FAASR-NH_2) was purchased from Synpep Corp.

Plasmids. Plasmid pEB-BFr13 is a vector that encodes SGPB with the mutations T213L and F228H (*22*). The resulting construct was used to produce a thermostable protease, SGPBFr13 (*22*). Plasmid pEB-HDa is a vector that encodes SGPB with the mutation S195A. This mutation of the catalytic triad results in an enzyme with reduced hydrolytic activity and increased peptide ligase activity (*21*). Plasmid pEB-HDaF13 was constructed by digesting pEB-HDa and pEB-BFr13 with *Sca*I and *Rsr*II restriction endonucleases. The 6 kb fragment from the pEB-HDa digestion was ligated with the 1 kb fragment from pEB-BFr13 with

[1] Abbreviations: CAPS, 3-(*N*-cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; pmP1, promature P1 site; SGPB, *Streptomyces griseus* protease B; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

T4 DNA ligase. The ligated construct was transformed into *E. coli* for amplification. Restriction analysis and DNA sequencing were used to verify the sequence. Verified constructs were transformed into *B. subtilis* for expression. Plasmid pEB-HDaF13 encodes for an SGPB variant that combines the peptide-ligation activity of the S195A variant with the stability of SGPBFr13.

Expression of Thermostable Peptide Ligase. A culture of *B. subtilis* that had been transformed with pEB-HDaF13 was diluted to 15 L with PEMC Kan (32 g/L tryptone, 2 g/L yeast extract, 5 g/L sodium chloride, 10 mM calcium chloride, 50 mg/L kanamycin). The 15 L culture was supplemented with 0.35 unit/L SGPD and incubated for 72 h at 30 °C with 6 L/min aeration and 200 rpm stirring in a Chemap fermenter (*21*).

Peptide Ligase Purification. The cellular component of the 15 L culture was removed via tangential flow filtering on a Millipore Pellicon apparatus that had been installed with 0.45 μm cutoff Durapore cartridges. The filtrate, which contained the desired protein, was applied to a Pellicon unit fitted with a 10000 NMWL (nominal molecular weight limit) polysulfone cartridge. The filtrate was concentrated down to 1.0 L and diafiltered against 8 L of exchange buffer (100 mM sodium acetate, pH 5.0, 2 mM calcium chloride) to a final volume of 1.5 L. The buffer-exchanged enzyme (400 mL) was loaded at 5 mL/min onto a Source 15Q anion-exchange column equilibrated with Q buffer (25 mM sodium acetate, pH 5.0, 2 mM calcium chloride). The enzyme, which eluted with the flow-through, was collected and applied to a Source 15S cation-exchange column equilibrated with Q buffer. The enzyme was eluted from the Source 15S resin at a 5 mL/min flow rate with a sodium chloride gradient of 1 mM/mL. Fractions that contained the enzyme were identified by SDS−PAGE analysis and pooled for dialysis into S buffer (5 mM sodium acetate, pH 4.6, 2 mM calcium chloride). The dialyzed sample was loaded onto a Source 15S column equilibrated with S buffer. The enzyme was eluted at a 5 mL/min flow rate with a sodium chloride gradient of 0.67 mM/mL. Fractions that contained the enzyme were identified by SDS−PAGE and Western blot analysis (*24*). The pure enzyme was pooled, dialyzed with S buffer, and stored at −20 °C.

Ligation Reactions. Standard ligation reactions were performed at 30 °C for 16 h in 24 μL of 40 mM Tricine, pH 8.0, containing 275 nmol of FAASF-NH_2, 30 nmol of Sc-AAPF-SBn (or Sc-AAPF-*p*NA), and 30 pmol of peptide ligase. The efficiency of these ligation reactions was analyzed by using a Waters 991 Controller/600 Detector HPLC unit coupled with a reverse-phase Waters 8 × 100 mm Prep Nova-Pak HR C_{18} Radial-Pak column. Solvent A consisted of 0.05% TFA in water. Solvent B was acetonitrile with 0.045% TFA. Products of the ligation reactions were eluted with a gradient of 5−65% solvent B over 30 min. Peaks from HPLC analysis were identified by retention times and liquid secondary ion mass spectrometry (LSIMS). Ligation reactions were performed in guanidinium chloride solutions where the final concentrations of guanidinium chloride were 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 M. In addition, ligations in sodium dodecyl sulfate (SDS) were performed under the standard ligation conditions with SDS concentrations of 0.05%, 0.1%, and 0.5% (w/v). All ligation to hydrolysis ratios are corrected for background (noncatalyzed) hydrolysis of the thiobenzyl ester acyl donor. No background hydrolysis was observed under any ligation conditions for the *p*-nitroanilide acyl donor.

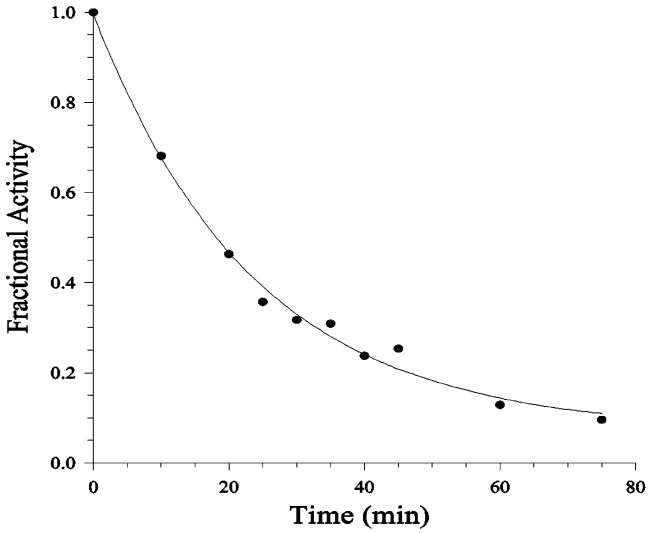


FIGURE 1: Time course for loss of ligase activity exhibited by streptoligase when incubated at 55 °C in the absence of substrate. The line through the data points is the best first-order fit to the data.

Thermostability. The effect of temperature on activity was determined by heating the peptide ligase in Tricine buffer to 55 °C for various time periods, after which the solution was cooled (ice/water bath) and remaining ligase activity was assayed using the standard ligation protocol.

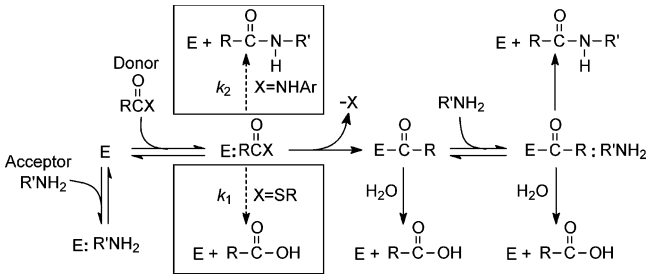
pH−Ligation Profile. To determine the effect of pH on ligation, measurements were carried out over a pH range of 5.5−9.5. The buffers were used were MES (pH range 5.5−6.0), MOPS (pH range 7.0−7.5), Tricine (pH range 8.0−8.5), and CHES (pH range 9.0−9.5).

RESULTS AND DISCUSSION

Thermostability of Streptoligase. Streptoligase, the triple mutant of SGPB (S195A, T213L and F228H), combines the characteristics of (i) the peptide-ligating enzyme described by Elliott et al. (*21*) and (ii) the stabilizing mutations described by Sidhu et al. (*22*). A preliminary experiment showed that streptoligase was able to catalyze the coupling of a thioester donor (Sc-AAPF-SBn) and an amine acceptor (FAASF-NH_2) to give a nonapeptide (data not shown). Following this initial success, the thermostability of streptoligase was tested at 55 °C, and the decrease in its ligase activity was monitored as a function of time. Shown in Figure 1 is the plot of fractional ligase activity versus time. The derived first-order rate constant for this process is $(7.1 \pm 0.6) \times 10^{-4}$ s^{-1}.

The two substitutions (T213L and F228H) cause a modest, 10-fold, increase in the half-life for activity of SGPB at 55 °C (*22*), a value that is equivalent to a change in the activation free energy for unfolding ($\Delta\Delta G^{\ddagger}$) of 6.1 kJ mol^{-1}. Furthermore, this value is consistent with that expected for the introduction of an additional buried hydrogen bond (*25*). In the context of the ligase SGPB S195A, these two substitutions result in a greater than additive increase in the protein's thermostability. That is, the SGPB S195A variant is completely inactivated in 1 min at 40 °C (*21*), whereas streptoligase only loses half its activity in 16.3 min at 55 °C

Scheme 1



(Figure 1). If it is assumed, conservatively, that a residual activity of 5% would have been observed in inactivation experiments involving the SGPB S195A variant, the rate of inactivation of this enzyme at 40 °C can be estimated at greater than 0.05 s^{-1}. Using the Eyring equation, the $\Delta G^{\ddagger}$ for unfolding of the S195A variant is less than 84.6 kJ mol^{-1} at 40 °C, and the corresponding $\Delta G^{\ddagger}$ for unfolding of streptoligase is 100.4 kJ mol^{-1} at 55 °C. Therefore, the change in free energy of inactivation ($\Delta\Delta G^{\ddagger}$) between the SGPB S195A variant and streptoligase is greater than 15.8 kJ mol^{-1}, a free energy difference that is more than two and one-half times that seen for stabilization of the wild-type enzyme on incorporation of the same two binding site mutations (T213L and F228H). Indeed, streptoligase has roughly 2-fold greater stability than the wild-type protease SGPB.

Mechanistic Studies on Streptoligase. The mechanism proposed by Elliott et al. for the SGPB S195A variant peptide ligase is shown in Scheme 1. According to this scheme the enzyme intermediate [E−C(O)R] is acylated on the active site histidine, and the two "boxed" pathways are not kinetically important (*21*).

This mechanistic scheme was based on several key observations: (i) at pH values below 6.5, the total activity of the enzyme drops dramatically; (ii) the rate of substrate hydrolysis catalyzed by the SGPB S195A variant (k_{cat}/K_m) varies according to the intrinsic substrate reactivities. That is, thioesters react faster than esters, which in turn react more rapidly than *p*-nitroanilides; (iii) at a given acceptor concentration, the ratio of aminolysis to hydrolysis is independent of donor substrate activity; and (iv) at high acceptor concentrations the total activity of the enzyme decreases (*21*).

Similarities between the Mechanisms of Action of Streptoligase and the SGPB S195A Variant. The pH−activity profile for streptoligase is shown in Figure 2. The pH profiles of streptoligase and the SGPB S195A variant (*21*) are remarkably similar. The pH−activity profile of streptoligase is in keeping with a mechanism involving acylation of the active site histidine followed by acyl transfer to either a water or an amine nucleophile. In particular, the ratio of aminolysis to hydrolysis increases with pH before plateauing at pH values greater the 8.5 (Figure 2), an observation that suggests aminolysis occurs via attack on the acyl-enzyme intermediate by a neutral amine nucleophile.

The total activity of streptoligase and the SGPB S195A variant is attenuated at pH values greater than 6.5. The reason behind this observation is unclear and could involve a deprotonation event remote from the active site.

Notably, the absolute rates of ligation and hydrolysis decrease with increasing acceptor peptide (FAASF-NH_2) concentration, an observation that is consistent with the

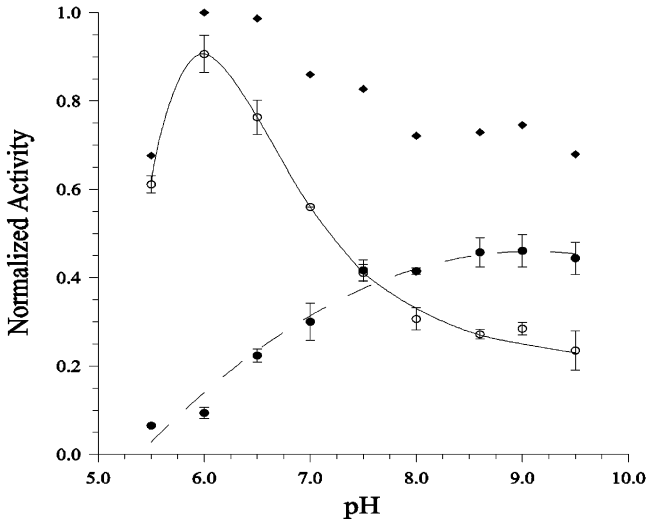


FIGURE 2: Effect of pH on the streptoligase-catalyzed ligation (●) and hydrolysis (○) reactions. Also shown is the total enzymatic activity (◆). Error limits are encompassed within the symbol diameter except where shown explicitly. Trend lines are for the visual aid of the reader.

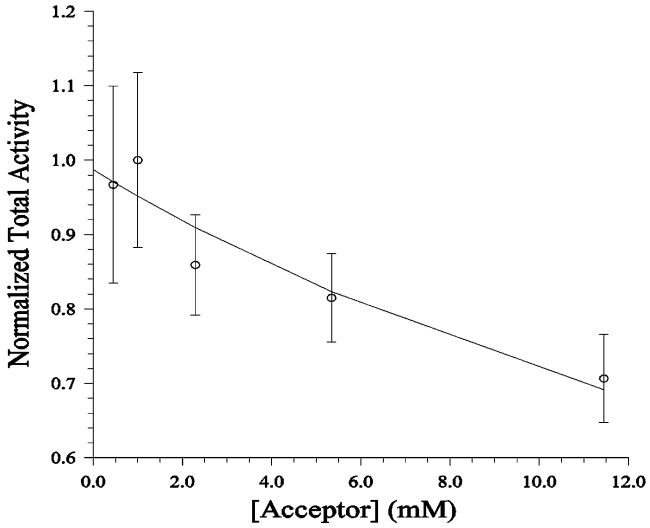


FIGURE 3: Effect of acceptor nucleophile concentration (NH_2-FAASR-NH_2) on the total streptoligase activity. The included line is the best fit to a competitive inhibition model.

acceptor (R′NH_2, Scheme 1) binding in competition to the donor substrate (Sc-AAPF-SBn) in the enzyme's active site (Figure 3). In the present case, the inhibition of streptoligase activity by the acceptor peptide FAASF-NH_2 is less pronounced than that reported for the SGPB S195A variant [50% inhibition of activity at 4.0 mM acceptor (*21*)].

Differences between the Mechanisms of Action of Streptoligase and the SGPB S195A Variant. In contrast to the situation with the SGPB S195A variant at 30 °C, the streptoligase-catalyzed ratio of ligation to hydrolysis is dependent on the leaving group (Table 1). In fact, this relationship was seen at a range of temperatures (Table 2).

Therefore, the mechanism outlined in Scheme 1 must be modified to incorporate one of the following two possibilities: (i) streptoligase also catalyzes hydrolysis of the thioester bond in Sc-AAPF-SBn, or (ii) streptoligase catalyzes aminolysis of the amide bond in Sc-AAPF-*p*NA as well as acylation of the active site histidine. Given that the two newly introduced mutations (T213L and P228H) are in the P1

Table 1: Ratio of Ligation to Hydrolysis for Streptoligase-Catalyzed and SGPB S195A Variant-Catalyzed Reactions with Two Separate Donors at 30 °C

enzyme	donor peptide	ligation:hydrolysis product ratio
SGPB S195A variant[a]	Sc-AAPF-SBn	1.80
	Sc-AAPF-*p*NA	1.68
streptoligase	Sc-AAPF-SBn	1.4 ± 0.2
	Sc-AAPF-*p*NA	2.6 ± 0.5

[a] Data taken from ref *21*.

Table 2: Ratio of Ligation to Hydrolysis for Streptoligase-Catalyzed Reactions with Two Separate Donors at Various Temperatures

temp (°C)	Sc-AAPF-SBn	Sc-AAPF-*p*NA
20		3.3 ± 0.2
30	1.4 ± 0.2	2.6 ± 0.5
37	1.2 ± 0.3	2.2 ± 0.6
45	0.90 ± 0.08	2.0 ± 0.1
50	0.84 ± 0.09	1.7 ± 0.2
60	0.67 ± 0.08	1.9 ± 0.5

binding site, it is probable that streptoligase-catalyzed hydrolysis of Sc-AAPF-SBn occurs via a mechanism in which the histidine acts as a general base to assist the direct attack of water on the thioester carbonyl carbon (k_1 pathway in Scheme 1). The alternative possibility, a direct amine attack on the bound anilide substrate (k_2 pathway in Scheme 1), is less likely. Reactions that have lower activation energies (k_1, hydrolysis of thioesters) are more readily catalyzed than those with higher thermodynamic barriers (k_2, aminolysis of amides) (*26*).

A positive feature of streptoligase as a ligation catalyst is that it can be used with *p*-nitroanilides, a series of donors in which no background hydrolysis occurs under any of the ligation conditions studied. Shown in Figure 4 are the relative rates of streptoligase-catalyzed hydrolysis and ligation of Sc-AAPF-*p*NA at elevated temperatures. Notably, at the maximal ligase activity (50 °C), over 60% of the product is coupled peptide.

An inherent advantage of streptoligase is its ability to ligate peptides under denaturing conditions. For example, in the reaction involving Sc-AAPF-SBn in 0.2% w/v SDS the ratio of ligation to hydrolysis is approximately 1 (Figure 5); moreover, significant ligation activity is observed even at 4 M guanidine hydrochloride (Figure 6).

Structural Basis for the Increased Thermostability of Streptoligase. Shown in Figure 7 is the structure of SGPB taken from the protein database with the bound inhibitor having been removed from view (*27*). The colored residues are (i) the catalytic triad in blue, (ii) the two mutation sites (Phe228 and Thr213) in cyan, (iii) residues that are spatially close to the mutation sites (Ile181, Thr183, and Thr226) in pink, and (iv) the two protein disulfide bonds in yellow. The increase in thermal stability most likely results from increased interactions between the various protein folds. For example, the O−H group in Thr183 is 3.27 Å away from one of the two ϵ-carbon atoms in Phe228, a position that is equivalent to the ϵ-nitrogen atom in histidine. As a result, the mutation F228H could introduce a stabilizing H-bonding interaction between residues 228 and 183.

Wells and co-workers have extensively re-engineered the serine protease subtilisin to create a variety of useful catalysts

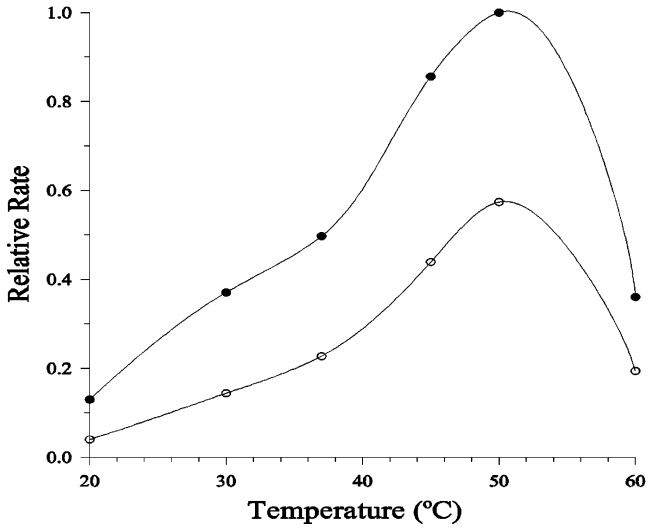


FIGURE 4: Effect of temperature on the streptoligase-catalyzed ligation (●) and hydrolysis (○) reactions. Trend lines are for the visual aid of the reader.

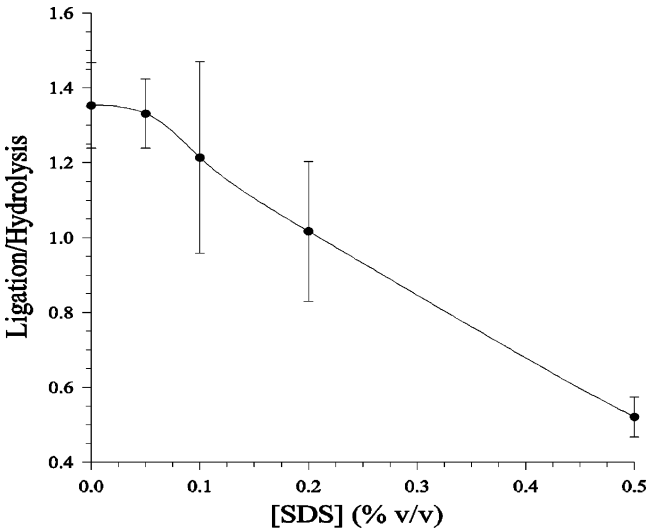


FIGURE 5: Effect of sodium dodecyl sulfate concentration (SDS) on the ratio of streptoligase-catalyzed ligation to hydrolysis. Trend lines are for the visual aid of the reader.

for a variety of different purposes. In an elegant series of studies these authors created a stable, peptide-ligating variant (derived from the precursor subtiligase) which is now known as stabiligase. Significantly, the subtilisin and chymotrypsin families of enzymes differ in terms of their protein fold, they have unique spectrums of substrate specificity, and they require unique sets of mutations to generate useful peptide-ligating variants.

Streptoligase is a member of a family of enzymes with diverse specificities including trypsin-like enzymes and enzymes with primary specificity for glutamic acid (*28*). Consequently, the mutational strategy described in this report could be applied to other enzymes of this family to generate a whole new class of ligase catalysts also possessing diverse activities and utility.

CONCLUSIONS

In summary, a triple mutant of a bacterial protease from *S. griseus* named streptoligase has been successfully made. This enzyme efficiently couples donor and acceptor peptides

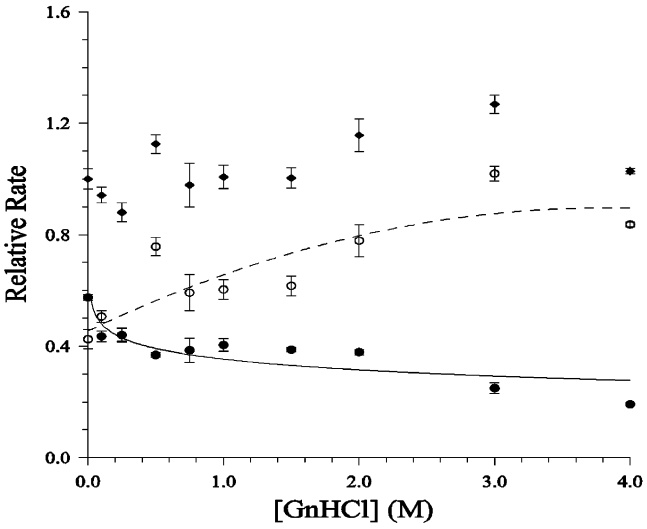


FIGURE 6: Effect of guanidinium concentration on the streptoligase-catalyzed ligation (●) and hydrolysis (○) reactions. Also shown is the total enzymatic activity (◆). Error limits are encompassed within the symbol diameter except where shown explicitly. Trend lines through the ligation and hydrolysis data are for the visual aid of the reader.

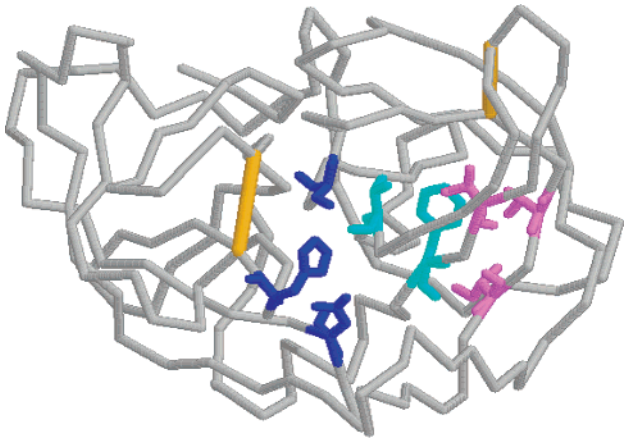


FIGURE 7: Three-dimensional structure of SGPB. Highlighted in this view are the catalytic triad in blue, the two stabilizing mutation sites in cyan, residues that are spatially close in pink, and the two protein disulfide bonds in yellow.

under a variety of conditions including both high temperatures (50 °C) and denaturing media (0.5% SDS or 4.0 M guanidine hydrochloride).

REFERENCES

1. Dawson, P. E., and Kent, S. B. (2000) Synthesis of native proteins by chemical ligation, *Annu. Rev. Biochem. 69*, 923−960.
2. Paulus, H. (2000) Protein splicing and related forms of protein autoprocessing, *Annu. Rev. Biochem. 69*, 447−496.
3. Van Maarseveen, J. H., and Back, J. W. (2003) Re-engineering the genetic code: Combining molecular biology and organic chemistry, *Angew. Chem., Int. Ed. Engl. 42*, 5926−5928.
4. Bordusa, F. (2002) Proteases in organic synthesis, *Chem. Rev. 102*, 4817−4868.
5. Dawson, P. E., Muir, T. W., Clark-Lewis, I., and Kent, S. B. (1994) Synthesis of proteins by native chemical ligation, *Science 266*, 776−779.
6. Nilsson, B. L., Kiessling, L. L., and Raines, R. T. (2000) Staudinger ligation: a peptide from a thioester and azide, *Org. Lett. 2*, 1939−1941.
7. Nilsson, B. L., Hondal, R. J., Soellner, M. B., and Raines, R. T. (2003) Protein assembly by orthogonal chemical ligation methods, *J. Am. Chem. Soc. 125*, 5268−5269.
8. Hirata, R., Nakano, A., Kawasaki, H., Suzuki, K., and Anraku, Y. (1990) Molecular-structure of a gene, Vma1, encoding the catalytic subunit of H^+-translocating adenosine-triphosphatase from vacuolar membranes of *saccharomyces-cerevisiae*, *J. Biol. Chem. 265*, 6726−6733.
9. Kane, P. M., Yamashiro, C. T., Wolczyk, D. F., Neff, N., Goebl, M., and Stevens, T. H. (1990) Protein splicing converts the yeast Tfp1 gene-product to the 69-Kd subunit of the vacuolar H^+-adenosine triphosphatase, *Science 250*, 651−657.
10. Giriat, I., and Muir, T. W. (2003) Protein semi-synthesis in living cells, *J. Am. Chem. Soc. 125*, 7180−7181.
11. Wang, L., Brock, A., and Schultz, P. G. (2002) Adding L-3-(2-naphthyl)alanine to the genetic code of *E. coli*, *J. Am. Chem. Soc. 124*, 1836−1837.
12. Chin, J. W., Santoro, S. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002) Addition of *p*-azido-L-phenylalanine to the genetic code of *Escherichia coli*, *J. Am. Chem. Soc. 124*, 9026−9027.
13. Murakami, H., Kourouklis, D., and Suga, H. (2003) Using a solid-phase ribozyme aminoacylation system to reprogram the genetic code, *Chem. Biol. 10*, 1077−1084.
14. Kwon, I., Kirshenbaum, K., and Tirrell, D. A. (2003) Breaking the degeneracy of the genetic code, *J. Am. Chem. Soc. 125*, 7512−7513.
15. Frankel, A., Millward, S. W., and Roberts, R. W. (2003) Encodamers: Unnatural peptide oligomers encoded in RNA, *Chem. Biol. 10*, 1043−1050.
16. Dedkova, L. M., Fahmi, N. E., Golovine, S. Y., and Hecht, S. M. (2003) Enhanced D-amino acid incorporation into protein by modified ribosomes, *J. Am. Chem. Soc. 125*, 6616−6617.
17. Ulijn, R. V., Moore, B. D., Janssen, A. E. M., and Halling, P. J. (2002) A single aqueous reference equilibrium constant for amide synthesis-hydrolysis, *J. Chem. Soc., Perkin Trans. 2*, 1024−1028.
18. Jackson, D. Y., Burnier, J., Quan, C., Stanley, M., Tom, J., and Wells, J. A. (1994) A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues, *Science 266*, 243−247.
19. Chang, T. K., Jackson, D. Y., Burnier, J. P., and Wells, J. A. (1994) Subtiligase: a tool for semisynthesis of proteins, *Proc. Natl. Acad. Sci. U.S.A. 91*, 12544−12548.
20. Atwell, S., and Wells, J. A. (1999) Selection for improved subtiligases by phage display, *Proc. Natl. Acad. Sci. U.S.A. 96*, 9497−9502.
21. Elliott, R. J., Bennet, A. J., Braun, C. A., Macleod, A. M., and Borgford, T. J. (2000) Active-site variants of *Streptomyces griseus* protease B with peptide-ligation activity, *Chem. Biol. 7*, 163−171.
22. Sidhu, S. S., and Borgford, T. J. (1996) Selection of *Streptomyces griseus* protease B mutants with desired alterations in primary specificity using a library screening strategy, *J. Mol. Biol. 257*, 233−245.
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Baardsnes, J., Sidhu, S., Macleod, A., Elliott, J., Morden, D., Watson, J., and Borgford, T. (1998) *Streptomyces griseus* protease B: secretion correlates with the length of the propeptide, *J. Bacteriol. 180*, 3241−3244.
25. Chen, Y. W., Fersht, A. R., and Henrick, K. (1993) Contribution of buried hydrogen bonds to protein stability. The crystal structures of two barnase mutants, *J. Mol. Biol. 234*, 1158−1170.
26. Bennet, A. J., and Brown, R. S. (1998) in *Comprehensive biological catalysis: a mechanistic reference* (Sinnott, M., Ed.) pp 293−326, Academic Press, San Diego, CA.
27. Read, R. J., Fujinaga, M., Sielecki, A. R., and James, M. N. (1983) Structure of the complex of *Streptomyces griseus* protease B and the third domain of the turkey ovomucoid inhibitor at 1.8-A resolution, *Biochemistry 22*, 4420−4433.
28. Sidhu, S. S., Kalmar, G. B., and Borgford, T. J. (1993) Characterization of the gene encoding the glutamic-acid-specific protease of *Streptomyces griseus*, *Biochem. Cell Biol. 71*, 454−461.

BI0496337