Surface Charge Substitutions Increase the Stability of α-Lytic Protease in Organic Solvents

Pascal Martinez and Frances H. Arnold*

Division of Chemistry and Chemical Engineering 210-41
California Institute of Technology
Pasadena, California 91125

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The ability to use enzymes in organic solvents greatly expands the potential applications of enzymes in organic synthesis. Most enzymes, however, are severely destabilized by the polar organic solvents of practical interest. To investigate the relationship between a protein's surface and its stability in nonaqueous solvents, four charged residues on the surface of α-lytic protease were replaced by most of the other possible amino acids. A total of seven sutstitutions at two of the four positions were found to improve stability in 84% dimethylformamide (DMF); six of the stabilizing substitutions were large, hydrophobic amino acids: Phe, Tyr, Ile, and Leu. A variant containing two surface substitutions is 27 times more stable than the wild-type enzyme in 84% DMF.

A protein's natural environment is often reflected in the composition of its surface. Surfaces of water-soluble proteins generally contain the bulk of the charged residues, where they are solvated by the aqueous medium and serve to solubilize the protein. On the other hand, charges are relatively rare on the membrane-exposed surfaces of membrane proteins and other proteins, such as crambin, which are soluble and stable in nonaqueous solvents.[1] Desolvation of surface charges can contribute to destabilization; for example, replacement of water by an organic solvent could require conformational changes that allow solvation of charged residues by polar groups in the protein.

A general strategy that has been proposed for stabilizing enzymes in nonaqueous media is to remove selected surface charges, replacing them with more hydrophobic amino acids.[1,2] To test this hypothesis, we have engineered the surface of α-lytic protease, a highly stable serine protease containing 198 amino acids and three disulfide bonds.[3] In organic solvents, α-lytic and other proteases are potentially useful for peptide synthesis by aminolysis of peptide esters or by reversal of the normal hydrolysis reaction.[4]

Charged surface residues targeted for replacement were those not implicated in catalysis and those judged least likely to participate in favorable noncovalent intramolecular interactions (salt bridges and hydrogen bonds). Of the 20 charged residues of α-lytic protease, four were chosen: Arg 45, Arg 78, Glu 125, and Arg 129 (numbering system of ref 5). To replace a targeted residue with as many of the remaining 19 amino acids as possible, site-directed mutagenesis with spiked oligonucleotides was performed on a synthetic gene (Martinez and Arnold, in preparation). At each position, plasmid DNA from 55-75 *Escherichia coli* colonies expressing active protease was sequenced to find between 14 and 18 variants (Table I).

Hydrolytic activities on the specific substrate succinyl-Ala-Ala-Pro-Ala-*p*-nitroanilide (sAAPA-pna) were measured in aqueous buffer after incubation of the lyophilized culture supernatants in 84% DMF at 30 °C. Although inactivation rates are not direct physical measurements of conformational stability, inactivation is the result of unfolding events that are controlled by the enzyme's conformational stability. Since folding requires the proenzyme,[6] unfolding and consequent inactivation of α-lytic protease in DMF are irreversible, and stability in the organic solvent can be determined by assaying residual activity following dilution into aqueous buffer. Seven substitutions at two positions, 45 and 78, significantly increased enzyme stability in 84% DMF (Table I). Minor stability enhancements were indicated for three variants at positions 125 and 129; most of the substitutions decreased stability. The side chains of Glu 125 and Arg 129 apparently participate in interactions that contribute to the stability of the folded protein and cannot be substituted without compromising its integrity.

The seven stabilized variants at positions 45 and 78 were purified to homogeneity, and deactivation rates were determined in aqueous buffer at 60 °C and in 84% DMF at 30 °C. The half-lives for deactivation, summarized in Table II, are consistent with the relative stabilities obtained from the rapid assays of the unpurified enzymes (Table I). In aqueous buffer at 60 °C, the inactivation rates of the majority of the variants were approximately equal to those of the wild-type enzyme; no substitution improved the stability of α-lytic protease by more than 20% in the aqueous buffer. In contrast, replacement of Arg 45 by Glu, Ser, Leu, and Ile stabilizes α-lytic protease in DMF. Leu, Tyr, and Phe 78 are all more stable than Arg 78. The double mutant Glu 45 + Leu 78 is more stable in 84% DMF than either single mutant, and its

Table I. Relative Stabilities in 84% (v/v) DMF (30 °C) of α-Lytic Protease Variants Identified at the Four Targeted Amino Acid Positions[a]

	position			
	45	78	125	129
decreasing stability ↓	Glu	Leu	Gln	Val
	Ser	Tyr	Asn	[Arg]
	Leu	Phe	[Glu]	Tyr
	Ile	[Arg]	Pro	Phe
	[Arg]	Lys	Ala	Thr
	Tyr	Ala	Arg	Ala
	Phe	His	Ser	Asn
	Ala	Val	Gly	His
	Gly	Gln	Ile	Ser
	Cys	Met	Leu	Gly
	Lys	Cys	Lys	Lys
	His	Ile	Phe	Pro
	Met	Trp	Thr	Gln
	Thr	Glu	Tyr	Ile
	Gln	Ser	Val	Leu
	Val	Gly	Asp	
	Trp	Thr	His	
		Asp		
		Asn		

[a] Square brackets indicate a wild-type sequence. Methods: Supernatants of 4-mL overnight *E. coli* cultures in MOPS media were lyophilized, and activities for hydrolysis of sAAPA-pna were measured in 0.05 M Tris·HCl, 0.1 M KCl, pH 8.75, at 30 °C after incubation for 1 h in 84% DMF/16% 0.05 M Tris·HCl, 0.1 M KCl, pH 8.75, 30 °C.

Table II. Half-Lives of Stabilized α-Lytic Protease Variants in Aqueous Buffer (0.05 M Tris·HCl, 0.1 M KCl, pH 8.75) (60 °C) and in 84% (v/v) DMF/16% Buffer (30 °C)[a]

	$t_{1/2}$, min	
variant	aqueous buffer (60 °C)	84% DMF (30 °C)
wild type	118	23
Glu 45	113	138
Ser 45	143	32
Leu 45	127	34
Ile 45	127	31
Leu 78	133	96
Tyr 78	123	71
Phe 78	129	59
Glu 45 + Leu 78	128	626

[a] Residual activities for hydrolysis of sAAPA-pna were measured in 0.05 M Tris·HCl, 0.1 M KCl, pH 8.75 at 30 °C after incubation in buffer or DMF. Half-lives were determined from plots of ln (residual activity) versus time. The estimated errors for the values of $t_{1/2}$ average 2% and range from 1 to 5%.

* Corresponding Author.

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$t_{1/2}$ is 27 times that of wild type.

Arg 45 and Arg 78 are isolated charges on the surface of α-lytic protease and do not appear to participate in stabilizing intramolecular interactions. Both residues are highly solvent accessible and tolerate numerous amino acid substitutions with little effect on enzyme stability in aqueous buffer. In contrast, the stability of α-lytic protease in DMF is significantly increased by seven surface substitutions, six of which are large, hydrophobic side chains. Similarly, replacement of a surface lysine by tyrosine improved the stability of an engineered subtilisin BPN′ in DMF.[7] It is commonly assumed that there is relatively little change in the solvent environment of a surface residue upon folding and, therefore, that surface amino acids contribute little to stability. However, the folded, biologically active state preferred in water may no longer be preferred in an organic solvent. Replacement of a charged side chain with a hydrophobic one enhances the compatibility between the surface of the folded protein and the solvent environment and may remove a potential driving force for formation of alternate (inactive) structures. The observed stabilization of the Glu 45 variant cannot, of course, be fully explained by this argument; proteins are complex, and interactions other than those with solvent can be introduced or perturbed by the surface mutations. Since protein surfaces are relatively tolerant of amino acid substitutions, the replacement of charged surface residues with hydrophobic ones may be a generally useful strategy for engineering enzymes that are stable in polar nonaqueous solvents.

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New Hypervalent σ-Selenanes with a Transannular Se–N Bond from *N*-Methyl-5*H*,7*H*-dibenzo[*b*,*g*][1,5]selenazocine

Hisashi Fujihara,*,† Hisatomo Mima,† Megumi Ikemori,‡ and Naomichi Furukawa*,†

Department of Chemistry, University of Tsukuba Tsukuba, Ibaraki 305, Japan
Tsukuba Research Laboratories, Eisai Co., Ltd. 5-1-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

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A number of hypervalent organosulfur compounds, sulfuranes, have been reported.[1] In contrast, much less is known about the chemistry of selenanes.[2] Recently, we reported the isolation of a new σ-bonded selenaza dication salt of *N*-methyl-5*H*,7*H*-dibenzo[*b*,*g*][1,5]selenazocine (**1**).[3] We have now found that either the reaction of **1** with *tert*-butyl hypochlorite (*t*-BuOCl) or the reaction of the selenoxide **2** of **1** with $SOCl_2$ gave the corresponding chloroselenane **3** containing a transannular Se–N bond, which was further converted into the methyl- or phenyl-substituted ammonioselenane upon treatment with $(CH_3)_2CuLi$ or $(C_6H_5)_2CuLi$. This is the first example of an isolable σ-ammonioselenane with a chlorine ligand or a methyl or phenyl group at the apical position, although analogous sulfur compounds of **3** have been reported.[4]

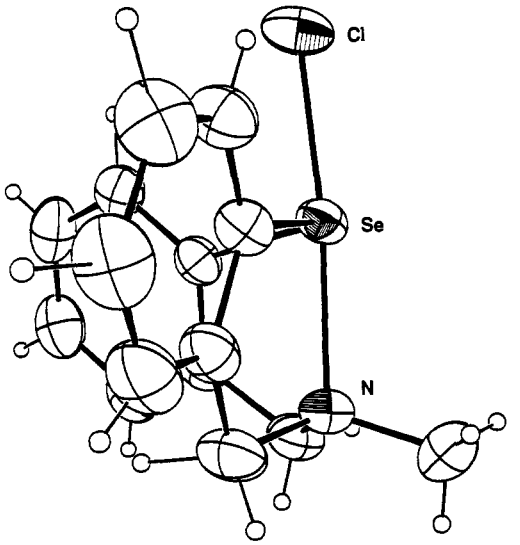
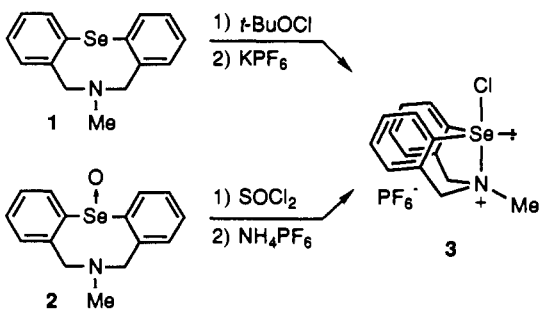


Figure 1. The crystal structure of **3**. The PF_6^- anion has been omitted for clarity.

Scheme I



Chloro-substituted σ-ammonioselenane **3** was isolated from the reaction of selenide **1** with *t*-BuOCl (Scheme I).[5] *t*-BuOCl is widely used as an oxidant for the conversion of selenides into selenoxides;[6] e.g., treatment of **1** first with *t*-BuOCl followed by alkaline hydrolysis with saturated sodium hydrogen carbonate solution gave exclusively the selenoxide **2** in 83% yield, contrasting with the oxidation of **1** using *m*-chloroperbenzoic acid, which gave a mixture of **2** and the *N*-oxide.[3] Although a chloroselenonium salt and/or hypervalent species have been proposed as intermediates in the reaction of diaryl selenides with *t*-BuOCl,[6] there is no example of isolation of its selenane.

Analogously, treatment of selenoxide **2** with $SOCl_2$ in anhydrous CH_2Cl_2 at room temperature followed by addition of NH_4PF_6

† University of Tsukuba.
‡ Eisai Co., Ltd.

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(5) (a) To a solution of **1**[3] (300 mg, 1.04 mmol) in anhydrous MeOH (5 mL) was added *t*-BuOCl (0.12 mL, 1.07 mmol) under an Ar atmosphere at −78 °C, and after 30 min the solution was allowed to reach 0 °C, followed by addition of KPF_6 and anhydrous CH_2Cl_2 (40 mL). Then the whole mixture was stirred at 0 °C for 21 h. Filtration under dry conditions and recrystallization from anhydrous CH_3CN–Et_2O gave a colorless crystal of **3** (65%). **3**: mp 178–180 °C dec. Anal. Calcd for $C_{15}H_{15}NClSePF_6$: C, 38.44; H, 3.23; N, 2.99. Found: C, 38.79; H, 3.19; N, 3.08. (b) In the above reaction with *t*-BuOCl, a trace amount (<10%) of *Se*-methoxy derivative **4** was produced after evaporation of the filtrate. **4**: mp 208.5–210.5 °C dec; ^{1}H NMR (CD_3CN) δ 2.85 (s, 3 H, NCH_3), 3.94 (s, 3 H, OCH_3), 4.31 (s, 4 H, CH_2), 7.36–7.53 (m, 2 H, Ar H), 7.53–7.72 (m, 4 H, Ar H), 7.92–8.14 (m, 2 H, Ar H); ^{13}C NMR (CD_3CN) δ 42.7, 56.6, 59.7, 128.7, 128.8, 130.8, 131.6, 133.6, 138.9; ^{77}Se NMR (CD_3CN) δ 828 (s); ^{31}P NMR (CD_3CN) δ −145.5 (sept, J_{PF} = 707 Hz). Anal. Calcd for $C_{16}H_{18}NOSePF_6$: C, 41.39; H, 3.91; N, 3.02. Found: C, 41.31; H, 3.75; N, 3.04.

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