THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 41, Issue of October 13, pp. 31635–31640, 2000

Printed in U.S.A.

Cold Adaptation of a Mesophilic Subtilisin-like Protease by Laboratory Evolution*

Received for publication, May 24, 2000, and in revised form, July 14, 2000
Published, JBC Papers in Press, July 21, 2000, DOI 10.1074/jbc.M004503200

Patrick L. Wintrode, Kentaro Miyazaki, and Frances H. Arnold‡

From the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Enzymes isolated from organisms native to cold environments generally exhibit higher catalytic efficiency at low temperatures and greater thermosensitivity than their mesophilic counterparts. In an effort to understand the evolutionary process and the molecular basis of cold adaptation, we have used directed evolution to convert a mesophilic subtilisin-like protease from *Bacillus sphaericus*, SSII, into its psychrophilic counterpart. A single round of random mutagenesis followed by recombination of improved variants yielded a mutant, P3C9, with a catalytic rate constant (k_{cat}) at 10 °C 6.6 times and a catalytic efficiency (k_{cat}/K_M) 9.6 times that of wild type. Its half-life at 70 °C is 3.3 times less than wild type. Although there is a trend toward decreasing stability during the progression from mesophile to psychrophile, there is not a strict correlation between decreasing stability and increasing low temperature activity. A first generation mutant with a >2-fold increase in k_{cat} is actually more stable than wild type. This suggests that the ultimate decrease in stability may be due to random drift rather than a physical incompatibility between low temperature activity and high temperature stability. SSII shares 77.4% identity with the naturally psychrophilic protease subtilisin S41. Although SSII and S41 differ at 85 positions, four amino acid substitutions were sufficient to generate an SSII whose low temperature activity is greater than that of S41. That none of the four are found in S41 indicates that there are multiple routes to cold adaptation.

The rates of the chemical reactions responsible for maintaining life are substantially reduced at the low temperatures encountered in polar regions or the deep ocean. Typically, the rate of a biochemical reaction decreases 2–3-fold when the temperature is lowered by 10 °C. The activity of an enzyme will thus be 16–80 times lower at 0 °C compared with 37 °C (1). Despite this, organisms native to cold environments achieve metabolic rates that are sufficient for survival and growth (2). Furthermore, enzymes isolated from cold-adapted organisms are generally more active at low temperatures (<10 °C) than their mesophilic homologs (1, 3, 4).

Uncovering the molecular basis of cold adaptation is of considerable interest for the insight it may provide into the nature of enzymatic catalysis as well as for potential biotechnology applications. Studies thus far have focused chiefly on comparisons of naturally occurring psychrophilic enzymes with their mesophilic counterparts. Such studies are complicated by the large evolutionary distances that separate natural homologs adapted to different temperatures. A large fraction of the amino acid substitutions may be neutral; others may reflect adaptation to other environmental conditions (*e.g.* high salt concentrations). Ignorance of the selective pressures under which the enzymes evolved further obscures attempts to identify specific adaptive mechanisms (5, 6). More recently, directed evolution has been used to investigate mechanisms of temperature adaptation. In particular, random mutagenesis combined with screening or selection has successfully generated cold-adapted variants from mesophilic enzymes (7–9).

Here we describe the directed evolution of low temperature activity in a mesophilic enzyme, subtilisin SSII, from the tropical bacterium *Bacillus sphaericus*.

SSII has a high degree of sequence similarity to other known subtilisins from psychrophilic (10–12), mesophilic (13–15), and thermophilic (16) sources (Fig. 1). SSII shows high sequence identity (77.4%) with a psychrophilic subtilisin, S41, from the Antarctic bacterium *Bacillus* TA41. SSII and S41 share several extended loop regions not found in other subtilisins. S41 contains a large number of hydrophilic residues, particularly 22 Asp residues, that are predicted to be located mainly on the surface of the enzyme (12). The inserted loop regions and the unusually large number of charged surface residues are thought to be characteristic of psychrophilic enzymes (1, 12). SSII also contains an unusually large number of Asp residues (19), many of them located at the same positions as in S41 (Fig. 1). Despite its high sequence similarity with S41, however, SSII displays no psychrophilic behavior; both its low temperature activity and high temperature stability are similar to those of mesophilic subtilisins such as BPN′.

In previous work (5), we increased the stability of the psychrophilic subtilisin S41 while simultaneously maintaining its activity at low temperature, demonstrating that high thermostability is not necessarily incompatible with high catalytic activity at low temperatures. To complement the evolution of psychrophilic S41 to function at high temperature, here we investigated the adaptation of mesophilic SSII to low temperatures. The particular screening strategy required an increase in the low temperature activity of the mesophilic enzyme while allowing stability to vary. In this way we could directly probe a hypothetical process by which a mesophilic enzyme would adapt to function at low temperatures. The extremely high identity of the mesophilic SSII with the natural psychrophilic S41 facilitates comparison of the solutions to the challenge of cold adaptation that are discovered in the laboratory with those that are found in nature.

* This work was supported by Procter & Gamble. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Division of Chemistry and Chemical Engineering, 210-41, California Institute of Technology, Pasadena, CA 91125. Tel.: 626-395-4162; Fax: 626-568-8743; E-mail: frances@cheme.caltech.edu.

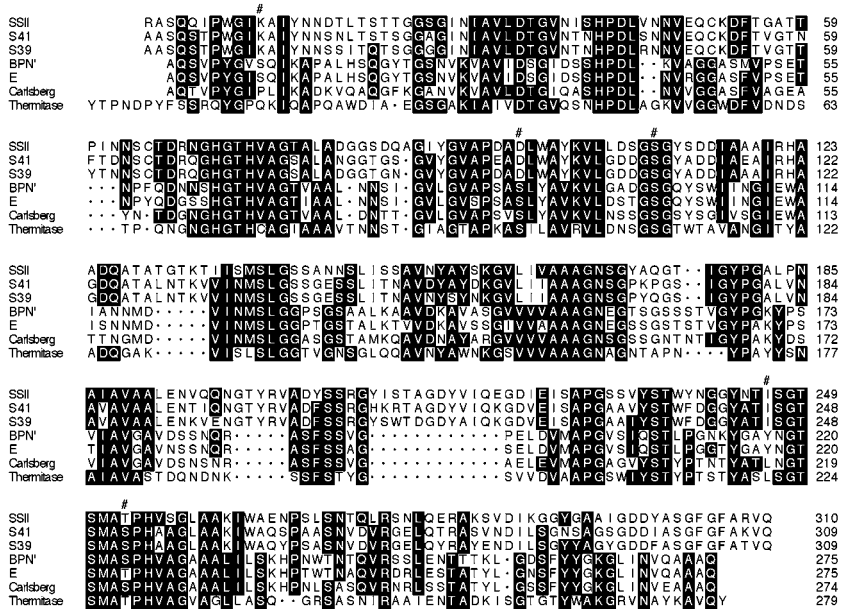

FIG. 1. **Multiple sequence alignment of subtilisins SSII (10), S41 (12), S39 (11), BPN′ (13), E (14), Carlsberg (15), and thermitase (16).** Conserved residues are *shaded*. The sequence alignment was constructed using CLUSTAL W (38).

EXPERIMENTAL PROCEDURES

Library Construction—Subtilisin SSII was expressed in *Bacillus subtilis* from the *Escherichia coli-Bacillus* shuttle vector pSPH2R that contains the prosequence of subtilisin S41 and the pre-prosequence of subtilisin BPN′ (17). The first generation library was generated by error-prone PCR.[1] SSII was amplified from pSPH2R with the primers 5′-GGATAACCAATTGTTCCTTGCGC-3′ and 5′-AAAGACTTTACAG-GTGCGACAAC-3′. The 100-μl reaction mixture contained 10 μl of 10× reaction buffer (10 mM Tris-HCl, 10 mM KCl, 1.5 mM $(NH_4)SO_4$, 0.1% (v/v) Triton® X-100) with 2.5 mM $MgCl_2$, 0.25 mM $MnCl_2$, 5 μl each of 4 mM dATP and dGTP, 5 μl each of 20 mM dTTP and dCTP, 5 μM each primer, ~5 ng of plasmid, and 5 units of *Taq* polymerase (Promega, Madison, WI). The PCR schedule was 2 min at 94 °C, followed by 14 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. PCR was carried out on an MJ Research (Watertown, MA) thermal cycler (PTC-200). The product was purified using Qiaquick™ (Qiagen, Santa Clarita CA) and restriction- digested with *Eco*RI and *Bam*HI. The digested product was purified and ligated into pSPH2R with T4 DNA ligase.

In vitro recombination of improved variants was accomplished using the staggered extension process (StEP) (18). The 50-μl reaction mixture contained 5 μl of 10× reaction buffer, 2.5 mM $MgCl_2$, 5 μl of 2 mM dNTP mix, 5 μM of each primer, 2 ng of each plasmid, 2.5 μl of H_2O, and 5 units of *Taq* polymerase (Promega). The PCR schedule consisted of 5 min at 95 °C followed by 70 cycles of 94 °C for 30 s and 55 °C for 5 s.

Screening of Mutant Libraries—In order to facilitate high efficiency transformation in *Bacillus*, an *E. coli* shuttle vector was employed (17). Ligations of library DNA were transformed into competent *E. coli* (strain HB101) by electroporation and grown on plates containing 50 μg/ml ampicillin. Colonies were scraped from the plates, and the DNA was purified using Qiagen minipreps. This DNA was used to transform competent *B. subtilis* (protease-deficient strain DB428). Transformation mixtures were grown on 50 μg/ml kanamycin. Single colonies were picked using sterile toothpicks and placed in 96-well plates containing 2× YT media (per liter: 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl) with 10 mM $CaCl_2$, 50 μg/ml kanamycin (1 ml per well) and grown in a shaking incubator for 48 h at 37 °C, 300 rpm. After growth, cells were pelleted by spinning at 5000 × *g,* and 10 μl of supernatant was transferred to replica 96-well plates. The replica plates were cooled in a 10 °C cold room for 15 min. After cooling, 100 μl of 50 mM Tris-HCl, 10 mM $CaCl_2$, 100 mM NaCl, 0.2 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF) (Sigma), pH 8.5, was added to the wells. The reaction was allowed to proceed for 1 min and then stopped by the addition of 100 μl of isopropyl alcohol. Product formation was assessed by reading the absorbance at 405 nm in a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Protein Purification—Transformed *Bacillus* cultures were grown in a shaking incubator at 37 °C, 300 rpm, in 1 liter of 2× YT medium with 10 mM $CaCl_2$ and 50 μg/ml kanamycin for 70 h. Cultures were chilled on ice and centrifuged at 5000 × *g* to pellet the cells. Supernatant was adjusted to 88 g/liter NaCl, 50 mM Tris, pH 7.6, and applied to a phenyl-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with 50 mM Tris-HCl, 88 g/liter NaCl, 2 mM $CaCl_2$, pH 7.6 (buffer A). The column was washed in buffer A until no further change in absorbance at 280 nm was seen. Protein was eluted with 50 mM Tris-HCl, 30 g/liter NaCl, 2 mM $CaCl_2$, 15% isopropyl alcohol, pH 7.6. Protein-containing fractions were pooled and concentrated to ~2 ml using an Amicon concentrator (Millipore, Burlington, MA) and dialyzed overnight against 2.5 liter of 50 mM Tris-HCl, 1.5 M NaCl, 10 mM $CaCl_2$, pH 7.6. Protein was then applied to a Superdex G-50 column and eluted at 0.5 ml/min. Fractions showing activity toward AAPF were pooled. Protein purity was estimated to be >95% by mass spectrometry.

Enzyme Activity Measurements—Proteolytic activity was determined on the small synthetic peptide substrate AAPF by monitoring the formation of released *p*-nitroaniline at 410 nm in a thermostatted Shimadzu (Columbia, MD) BioSpec-1601 spectrophotometer. The reaction buffer consisted of 50 mM HEPPS-NaOH (Calbiochem), pH 8.5, 100 mM NaCl, 10 mM $CaCl_2$. Concentration of the substrate was determined using an extinction coefficient ϵ_{315} = 14,000 M^{-1} cm^{-1} (19). Protein concentrations were estimated from the absorbance at 280 nm using an extinction coefficient ϵ_{280} = 47,578 M^{-1} cm^{-1} calculated according to Pace *et al* (20). Kinetic constants for wild-type SSII and mutants were determined from a series of initial rates at different concentrations of AAPF over the range of 0.02–1.4 mM that bracketed K_M. Reported values are the average of three measurements. The standard deviations do not exceed 10%.

Thermal Inactivation—Half-lives of irreversible thermal inactivation upon autolysis were determined at 70 °C in 50 mM HEPPS-NaOH, pH 8.5, 100 mM NaCl, 10 mM $CaCl_2$ using 2 μM enzyme. At various time intervals, 10-μl aliquots were removed and diluted into 1 ml of an assay solution (50 mM HEPPS-NaOH, pH 8.5, 100 mM NaCl, 10 mM $CaCl_2$, 1 mM AAPF) for the measurement of residual activity at 30 °C. Reported values are the averages of at least two measurements. The standard deviations do not exceed 10%. Dependence of thermal inactivation times at 60 °C on calcium concentration was determined using identical conditions except that the concentration of $CaCl_2$ in the enzyme solution was varied (concentrations were 0.005, 0.05, 0.5, 1.0, and 10 mM). Curves of $t_{1/2}$ *versus* [$CaCl_2$] were fit to the theoretical binding curve for a single site: $pCa^{2+} = pK_a + \log(E/E - Ca^{2+})$ (21).

Homology Modeling—A three-dimensional structural model of SSII was constructed based on its homology with subtilisins Carlsberg, Savinase, BPN′, and thermitase. Coordinates (Carlsberg, code 1CSE (22); BPN′, code 2SNI (23); thermitase, code 1TEC (24); and Savinase, code 1SVN (25)) were obtained from the Protein Data Bank (26). Sequence alignments and model construction and refinement were carried out using the homology module of the INSIGHT II molecular modeling software package (Biosym Technologies, San Diego, CA).

[1] The abbreviations used are: PCR, polymerase chain reaction; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; StEP, staggered extension process; AAPF, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide.

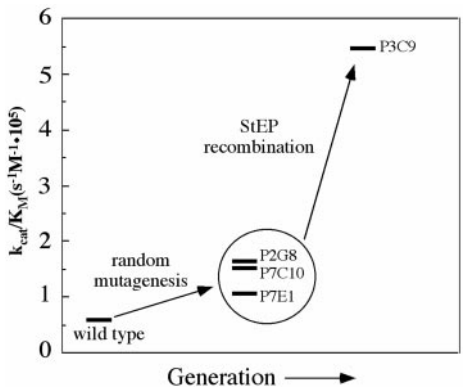

FIG. 2. **Progress of the evolution of low temperature activity in SSII.**

RESULTS

Evolution of Cold Activity in SSII—Screening ~3000 mutants from a first generation library prepared by error-prone PCR of SSII identified three variants (labeled P2G8, P7E1, and P7C10) with improved activity at 10 °C. StEP recombination of these variants and screening the resulting library yielded a fourth variant, P3C9, whose catalytic efficiency at 10 °C is ~9.6 times greater than wild type toward the peptide substrate AAPF (Fig. 2). Kinetic parameters for wild-type SSII and the four variants are given in Table I. Most of the observed increases in low temperature activity come from increases in k_{cat}; only P7C10 shows a significant decrease in K_M. The k_{cat} of the recombined variant P3C9 is ~6.6 times that of wild type. The k_{cat} of P3C9 is ~4.5 times that of S41 at 10 °C.

Mutations Present in Cold-active Variants—Amino acid substitutions found in the cold-active variants of SSII are listed in Table I. Five substitutions were found in the first generation variants, four of which are present in the recombination product P3C9. The absent mutation, Ile-246 → Leu, accompanied Lys-11 → Arg in P7E1 and may not be present in P3C9 because it is neutral with respect to cold activity. However, residue 246 is only seven amino acids away from 253, a mutation site in another cold-active variant. *In vitro* recombination methods such as StEP or DNA shuffling (27) often fail to recombine mutations that are so close to one another (28). It is therefore also possible that Ile-246 → Leu is cold-activating but is not found in P3C9 because it was not successfully recombined.

Evolution of Thermostability in SSII—Inactivation profiles at 70 °C for wild-type SSII and the stable variant p7E1 are shown in Fig. 3. Inactivation profiles were well fit by a single exponential, indicating that thermal inactivation is a first order process under the conditions employed. Half-lives at 70 °C are given in Table I.

Dependence of Thermostability on Calcium—Based on sequence homology with subtilisins of known structure, SSII is predicted to have two calcium-binding sites, a high affinity site that in other subtilisins is essential for activity (21) and a low affinity site that is important for thermal stability (21). Ligands for the low affinity site in SSII should include Ala-181 (Lys-170 in BPN′), Asp-223 (Glu-195 in BPN′), Glu-225 (Asp-197 in BPN′), Arg-275 (Arg-247 in BPN′), and Gln-279 (Glu-251 in BPN′). It has been noted that enzymes from psychrophilic organisms generally exhibit weaker affinity for stabilizing ions such as calcium than their mesophilic cousins (10, 29). Recent work has shown that the psychrophilic subtilisins S41 and S39 can both be stabilized significantly by increasing their affinity for calcium (5, 30). In order to probe the relationship between increased low temperature activity and calcium affinity in SSII, the half-life at 60 °C was determined as a function of calcium concentration for wild type and

TABLE I

Stability and activity parameters for SSII wild type and mutants

Half-lives at 70 °C and kinetic parameters at 10 °C for wild-type SSII and its cold-active variants were measured on the peptide substrate AAPF in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM HEPPS, pH 8.5. Standard deviations do not exceed 10%.

Variant	Amino acid substitution(s)	$t_{1/2}$ (70 °C)	k_{cat}	K_M
			s^{-1}	mM
Wild type		13.5	15.7	0.28
P2G8	T253A	11.0	61.1	0.39
P7E1	K11R I246L	19.2	34.8	0.33
P7C10	D98N S110F	5.7	21.0	0.14
P3C9	T253A K11R D98N S110F	4.1	104	0.19

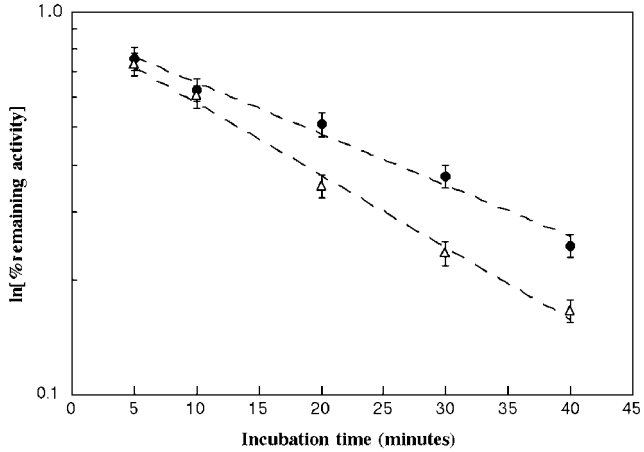

FIG. 3. **Loss of activity for wild-type SSII and mutant P7E1 upon incubation at 70 °C.** Symbols used are: △, wild type; ●, P7E1.

P3C9 (Fig. 4). As expected, increasing the calcium concentration decreases the rate of thermal inactivation. The dependence of half-life on calcium concentration follows a roughly sigmoidal curve. Assuming that the midpoint of the curve is related to the affinity of the enzyme for calcium (21), we see that the calcium affinity of P3C9 does not differ greatly from that of wild type (midpoints occur at *p*Ca values of 4.1 for wild type *versus* 3.8 for P3C9).

Temperature Dependence of Activity in Wild Type and P3C9—The specific activity of P3C9 relative to wild-type SSII is shown in Fig. 5 as a function of temperature. Kinetic parameters for wild type, P3C9, and the natural psychrophile S41 at different temperatures are given in Table II. P3C9 is more active than its mesophilic counterpart SSII over the entire range of temperatures where it is stable. However, the relative superiority of P3C9 over wild type is temperature-dependent. At 10 °C, P3C9 is 6.5 times more active than wild type, but this difference decreases with increasing temperature. At 60 °C, P3C9 is only ~3.4 times more active.

DISCUSSION

Mutants of SSII were screened solely for improvements in activity at low temperature. This was meant to simulate, in an approximate way, the presumed evolutionary pressure experienced by enzymes in natural psychrophilic organisms. The final product of the laboratory evolution, variant P3C9, had a k_{cat} 6.6 times greater and a k_{cat}/K_M 9.6 times greater than wild type at 10 °C. Furthermore, at 10 °C both k_{cat} and k_{cat}/K_M are larger than those of the natural psychrophilic homolog S41. Only four amino acid substitutions brought about this increase

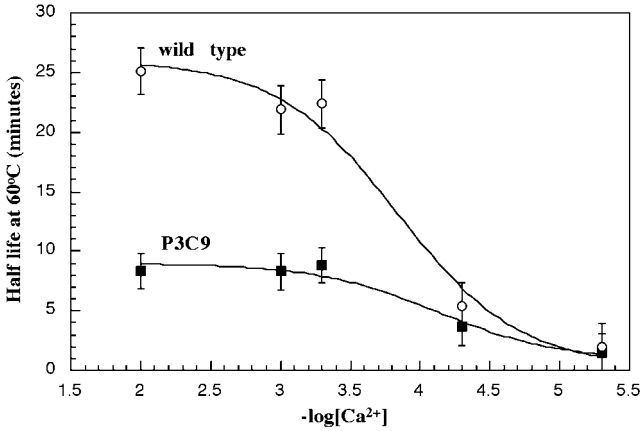

FIG. 4. **Dependence of thermal inactivation at 60 °C on $CaCl_2$ concentration for wild-type SSII and P3C9 in 100 mM NaCl, 50 mM HEPPS, pH 8.5.** Sigmoidal curves represent fits to a theoretical single binding site curve (see "Experimental Procedures").

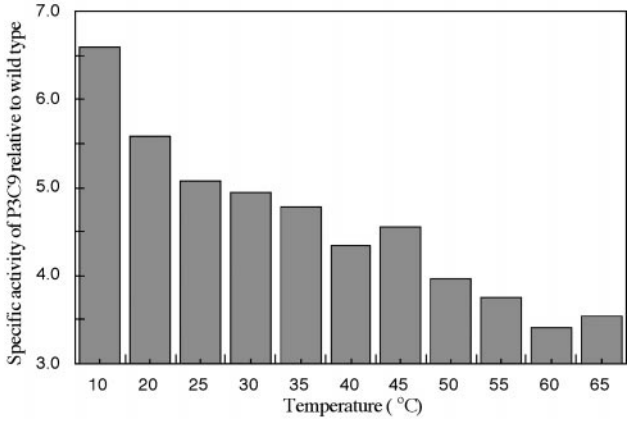

FIG. 5. **Ratio of the specific activities of P3C9 and wild-type SSII (10 mM $CaCl_2$, 100 mM NaCl, 50 mM HEPPS, pH 8.5) as a function of temperature.**

TABLE II
Temperature dependence of k_{cat} and K_M for SSII, P3C9, and S41

Kinetic parameters for wild-type SSII, recombined variant P3C9, and wild-type subtilisin S41 were measured on the peptide substrate AAPF at 10, 30, and 60 °C. Conditions were 10 mM $CaCl_2$, 100 mM NaCl, 50 mM HEPPS, pH 8.5. Standard deviations do not exceed 10%.

	k_{cat}	K_M	k_{cat}/K_M
	s^{-1}	mM	$\times 10^5\ M^{-1}\ s^{-1}$
10 °C			
Wild-type SSII	15.7	0.28	0.6
P3C9	104	0.19	5.5
S41	23.3	0.28	0.8
30 °C			
Wild-type SSII	90.0	0.34	2.6
P3C9	258	0.25	10.3
S41	63.8	0.27	2.4
60 °C			
Wild-type SSII	258	0.59	4.4
P3C9	579	0.53	10.9
S41	264	0.90	2.9

in cold activity, showing that SSII can rapidly adapt to low temperatures when strong selective pressure is applied. Particularly striking is the potential of single point mutations to give large increases in low temperature activity. Recent studies of natural psychrophiles have suggested that, despite the many differences observed between mesophilic and psychrophilic enzymes, single amino acid substitutions may be capable of conferring most psychrophilic characteristics (31, 32). In this case, a cold-active SSII can be generated by a single point mutation; the k_{cat} of the first generation mutant P2G8 is 3.9 times that of wild type and 2.6 times that of the natural psychrophile S41.

Comparison of wild-type and mutant activities at a single temperature is complicated by possible changes in substrate specificity during laboratory evolution on the nonnatural substrate AAPF. Also, the most distinctive feature of psychrophilic enzymes is not simply that they are more active than mesophiles but that they are specifically more active at low temperatures. Thus, of greater interest than the absolute value of the activity is the dependence of activity on temperature. The relative superiority of the activity of P3C9 over that of wild type is greatest at 10 °C and decreases with increasing temperature (Table II and Fig. 5). The effect of temperature on biological rate constants is often expressed in terms of the increase in k_{cat} that comes with raising the temperature 10 °C ("Q_{10}"). Q_{10} for wild-type SSII in the 10–30 °C range is ~2.8, which is typical for mesophilic enzymes (32). P3C9, in contrast, has a Q_{10} of ~1.2, similar to the Q_{10} of ~1.3 found for S41. Low values of Q_{10} are a common feature of cold-adapted enzymes (33). It has often been noted that naturally psychrophilic enzymes maintain catalytic rates at low temperatures that are comparable to those of mesophilic enzymes at moderate temperatures. This has been achieved in P3C9, whose k_{cat} of 104 s^{-1} at 10 °C is close to the k_{cat} of 90 s^{-1} at 30 °C for wild type. Based on these criteria, we may assert that, with P3C9, we have evolved a truly psychrophilic enzyme.

In common with natural psychrophilic enzymes, P3C9 is less thermostable than its mesophilic counterpart, wild-type SSII. Because P3C9 was evolved in the laboratory, we have access to all of the intermediate species generated during the course of evolution from mesophile to psychrophile. This allows us to examine the *process* by which the final combination of low temperature activity and high temperature stability was reached. From the half-lives reported in Table I we see that there is not a strict inverse relation between stability and low temperature activity. Although two of the first generation mutants were less stable than wild type, the variant P7E1, which shows a nearly 2-fold increase in k_{cat}/K_M at 10 °C, is actually more stable. This is consistent with the results of previous directed evolution of the psychrophilic subtilisin S41, in which stability ($t_{1/2}$) was increased ~500-fold with no loss of low temperature activity (5). The recombinant of all three SSII mutants, variant P3C9, is less stable than any of the first generation variants. We propose that these data are best explained by random drift rather than an intrinsic trade off between stability and low temperature activity. Because most mutations are destabilizing (34), the accumulation of multiple mutations, cold-activating or otherwise, will eventually destabilize an enzyme in the absence of selective pressure to maintain stability. Even without such selective pressure, a stabilizing mutation may occasionally be discovered, as in variant P7E1. However, such events will be uncommon, and stability will ultimately decrease due to the accumulation of multiple destabilizing mutations.

Another possible explanation for the generally low stability of psychrophilic enzymes is negative selection. This explanation asserts that highly stable enzymes will be resistant to turnover by normal cellular degradation mechanisms and may therefore accumulate and ultimately be harmful to the organism (31, 32, 35). This is unlikely to be the case with subtilisins, since they are naturally extracellular proteases. Although we cannot rule out the possibility of negative selection toward stability in cold-adapted enzymes, our results demonstrate that it is not *necessary* to invoke negative selection to explain low stability.

We note that, in the discussion above, "stability" does not

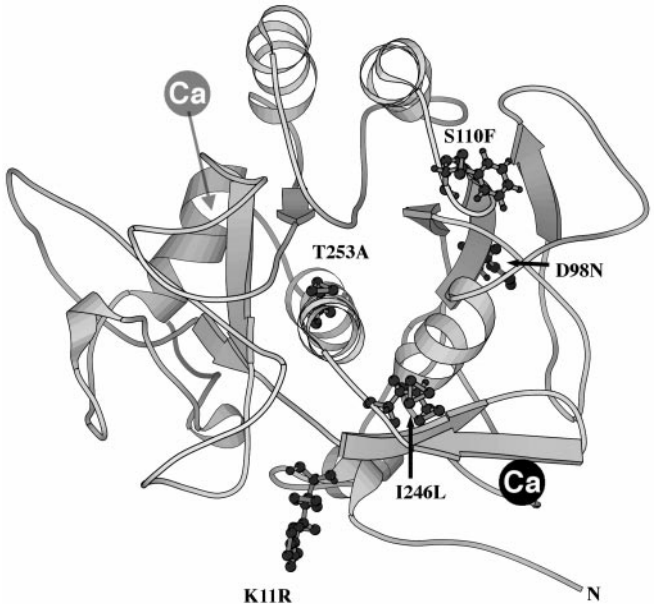

FIG. 6. **MolScript (39) diagram of a homology model of SSII showing the positions of the amino acid substitutions present in cold-active mutant P3C9.**

refer to the thermodynamic stability of the folded state (*i.e.* ΔG_{unfold}). Subtilisins unfold irreversibly, and thermodynamic parameters are thus not available. We have used instead the half-time of inactivation at high temperature, an effective measure of stability that is widely used in studies of subtilisins (7, 12, 21). In the specific case of subtilisin BPN′, it has been shown that the resistance to inactivation correlates with thermodynamic stability over a wide range of conditions (21).

Recently, several studies have appeared in which random mutagenesis and screening/selection were used to increase the activity of enzymes at temperatures below the natural physiological temperature. Two of these involved increasing the activity of hyperthermophilic enzymes at mesophilic temperatures (8. 9), whereas one increased the activity of a mesophilic subtilisin (BPN′) at 10 °C (7). Taguchi *et al.* (7) increased the low temperature (10 °C) activity of BPN′ by 70% using a mutagenesis/screening system. The activity increase was primarily due to an increased k_{cat}. Their improved triple mutant showed no loss of stability relative to wild type BPN′. None of the sites mutated in the cold-active BPN′ match sites found in this study.

Because the naturally psychrophilic S41 shares such high sequence identity with SSII, one clear route to cold adaptation would be to acquire cold-activating mutations present in S41. However, none of the mutations present in P3C9 are found in S41. Furthermore, except for Thr-253 → Ala, the mutations occurred at sites that are conserved between S41 and SSII. This result can be rationalized on several grounds. The space of all possible sequences for a 310-amino acid protein is vast, and there are likely to be multiple routes to cold adaptation. Additionally, the selective pressures applied during this work were undoubtedly different than those encountered by subtilisin in nature. For example, the synthetic peptide AAPF is not the natural substrate for either SSII or S41. Furthermore, our selection criteria were very stringent; only variants showing improvements of ~20% or greater were allowed to proceed to the next generation. In natural evolution, much smaller improvements could become fixed in the evolving population.

The three-dimensional structure of SSII is not known. However, we have constructed a model based on the homology of SSII with subtilisins of known structure (Fig. 6). The cold-activating mutations are distributed throughout the structure. There are substitutions at both the surface and buried positions and both close to and far from the active site. There is no mutation in or near the putative weak calcium-binding site, which is consistent with the observation that P3C9 has not lost its affinity for calcium.

In the absence of actual crystallographic or NMR data, we cannot identify the specific mechanisms responsible for the observed increase in low temperature activity. However, we can offer some tentative explanations. Thr-253 is located in a region that has high identity with subtilisin E (Fig. 1). The corresponding residue in subtilisin E, Thr-224, forms a hydrogen bond with Thr-220 (Thr-249 in SSII) adjacent to the catalytic serine. Disruption of this hydrogen bond by the replacement of threonine with a nonpolar residue such as alanine could lead to structural rearrangements or increased mobility in the active site. Such a disruption may be responsible for the large increase in low temperature activity. The Ser-110 → Phe substitution occurs near the entrance to the active site and replaces a polar amino acid with a nonpolar one. Residue 110 in SSII is equivalent to residue 101 in subtilisin BPN′ (Fig. 1), which is located in the S3 region of the binding pocket and is thought to interact with the P3 side chain of substrate molecules (36). Since Ser-110 → Phe occurs in the only mutant with a lower K_M (P7C10), it is possible that this mutation improves substrate binding through hydrophobic interactions with the highly nonpolar substrate. Asp-98 → Asn, the other mutation present in P7C10, is located on the opposite side of the protein from the active site. This mutation may be neutral or it may contribute to the observed improvement in the activity of P7C10. From the model, no explanation for an effect on activity can be offered. Lys-11 → Arg is one of two mutations found in P7E1, and the only one of these that is retained in P3C9. Based on the approximately additive increase in k_{cat} that results from the recombination of Lys-11 → Arg with Thr-253 → Ala, it appears that Lys-11 → Arg is largely responsible for the k_{cat} increase seen in P7E1. It is not clear, however, how the Lys-11 → Arg mutation acts to achieve this increase.

A recent study of temperature adaptation in lactate dehydrogenase A_4 (37) found that the cold-adapted enzymes possessed both higher k_{cat} and K_M values than their mesophilic homologs. This observation was rationalized in terms of localized increases in conformational flexibility; mutations that reduce the energetic barriers between different active site conformations (thus allowing for more rapid interconversion between them) will lead to higher values of k_{cat}. These same mutations, however, will allow the protein to more easily populate conformations that bind the substrate poorly, leading to increases in K_M. There is some support for this hypothesis in the present study. The two k_{cat} mutants in the first generation, P7E1 and P2G8, both show increases in K_M, and the mutant with the larger increase in k_{cat} also shows a larger increase in K_M (Table I). Furthermore, as mentioned above, mutation Thr-253 → Ala likely increases mobility in the active site. The results of recombination with mutant P7C10, however, demonstrate that deleterious effects on K_M values can be reversed by additional mutations. The increased stability of P7E1, despite its increases in both k_{cat} and K_M, suggests that such localized increases in conformational mobility need not be accompanied by decreases in global stability. In this our study agrees with the work on lactate dehydrogenase A_4, in which no correlation between adaptation temperature and stability at 50 °C was seen (37).

Acknowledgments—We are grateful to Professor Alan Porter (National University of Singapore) for providing the gene for wild-type SSII. We also thank Francisco Valles for technical assistance.

REFERENCES

1. Gerday, C., Aittaleb, M., Arpigny, J. L., Baise, E., Chessa, J. P., Garsoux, G., Petrescu, I., and Feller, G. (1997) *Biochim. Biophys. Acta* **1342,** 119–131
2. Hochachka, P. W., and Somero, G. N. (1984) *Biochemical Adaptation,* Princeton University Press, Princeton, NJ
3. Kristjansson, M. M., Bjarni, A., and Bjarnason, J. B. (1997) in *Food Proteins and Lipids* (Damaodaran, S., ed) Plenum Publishing Corp., New York
4. Jaenicke, R. (1990) *Philos. Trans. R. Soc. Lond-Biol. Sci.* **326,** 535–553
5. Miyazaki, K., Wintrode, P. L., Grayling, R. A., Rubingh, D. N., and Arnold, F. H. (2000) *J. Mol. Biol.* **297,** 1015–1026
6. Wintrode, P. L., and Arnold, F. H. (2000) *Adv. Protein Chem.* **55,** 161–225
7. Taguchi, S., Ozaki, A., Nonaka, T., Mitsui, Y., and Momose, H. (1999) *J. Biochem.* (*Tokyo*) **126,** 689–693
8. Merz, A., Yee, M., Szadowski, H., Pappenberger, G., Crameri, A., Stemmer, W. P., Yanofsky, C., and Kirschner, K. (2000) *Biochemistry* **39,** 880–889
9. Lebbink, J. H., Kaper, T., Bron, P., van der Oost, J., and de Vos, W. M. (2000) *Biochemistry* **39,** 3656–3665
10. Wati, M. R., Thanabalu, T., and Porter, A. G. (1997) *Biochim. Biophys. Acta* **1352,** 56–62
11. Narinx, E., Devail, S., Feller, G., and Gerday, C. (1992) *Biochim. Biophys. Acta* **1131,** 111–113
12. Davail, S., Feller, G., Narinx, E., and Gerday, C. (1994) *J. Biol. Chem.* **269,** 17448–17453
13. Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A., and Chen, E. Y. (1983) *Nucleic Acids Res.* **11,** 7911–7925
14. Stahl, M. L., and Ferrari, E. (1984) *J. Bacteriol.* **158,** 411–418
15. Jacobs, M., Eliasson, M., Uhlen, M., and Flock, J. I. (1985) *Nucleic Acids Res.* **13,** 8913–8926
16. Meloun, B., Baudys, M., Kostka, V., Hausdorf, G., Froemmel, C., and Hoehne, W. E. (1985) *FEBS Lett.* **183,** 195–200
17. Miyazaki, K., and Arnold, F. H. (1999) *J. Mol. Evol.* **49,** 716–720
18. Zhao, H., Giver, L., Affholter, J., and Arnold, F. H. (1998) *Nat. Biotechnol.* **14,** 258–261
19. DelMar, E. G., Largman, C., Brodick, J. W., and Geokas, M. C. (1979) *Anal. Chem.* **99,** 316–320
20. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, V. (1995) *Protein Sci.* **4,** 2411–2423
21. Pantoliano, M. W., Whitlow, M., Wood, J. F., Rollence, M. L., Finzel, B. C., Gilliland, G. L., Poulos, T. L., and Bryan, P. N. (1988) *Biochemistry* **27,** 8311–8317
22. Bode, W., Papamokos, E., and Musil, D. (1987) *Eur. J. Biochem.* **166,** 673–692
23. McPhalen, C. A., and James, M. N. G. (1988) *Biochemistry* **27,** 6582–6598
24. Gros, P., Betzel, C., Dauter, Z., Wilson, K. S., and Hol, W. G. J. (1989) *J. Mol. Biol.* **210,** 347–367
25. Betzel, C., Teplyakov, A. V., Harutyunyan, E. H., Saenger, W., and Wilson, K. S. (1990) *Protein Eng.* **3,** 161–172
26. Bernstein, F., Koetzle, T., Williams, G., Meyer, E. J., Brice, M., Rodgers, J., Kennard O., Shimanouchi, T., and Tasumi, M. (1977) *J. Mol. Biol.* **112,** 535–542
27. Stemmer, W. P. C. (1994) *Nature* **370,** 389–391
28. Volkov, A., Shao, Z., and Arnold, F. H. (2000) *Methods Enzymol.,* in press
29. Feller, G., Payan, F., Theys, F., Qian, M., Haser, R., and Gerday, C. (1994) *Eur. J. Biochem.* **222,** 441–447
30. Narinx, E., Baise, E., and Gerday, C. (1997) *Protein Eng.* **10,** 1271–1279
31. Somero, G. N. (1995) *Annu. Rev. Physiol.* **57,** 43–68
32. Feller, G., and Gerday, C. (1997) *Cell Mol. Life Sci.* **53,** 830–841
33. Low, P. S., Bada, J. L., and Somero, G. N. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 430–432
34. Giver, L., Gershenson, A., Freskgard, P. O., and Arnold, F. H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12809–12813
35. Benner, S. A. (1989) *Chem. Rev.* **89,** 789–806
36. Siezen, R. J., and Leunissen, J. A. M. (1997) *Protein Sci.* **6,** 501–523
37. Fields, P. A., and Somero, G. N. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11476–11481
38. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
39. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950