# Chemical Modification of Cysteine Mutants of Subtilisin *Bacillus lentus* Can Create Better Catalysts Than the Wild-Type Enzyme

Per Berglund,† Grace DeSantis,† Michele R. Stabile,† Xiao Shang,† Marvin Gold,‡ Richard R. Bott,§ Thomas P. Graycar,§ Tony Hing Lau,§ Colin Mitchinson,§ and J. Bryan Jones*,†

*Departments of Chemistry and of Molecular and Medical Genetics*
*University of Toronto, 80 St. George Street*
*Toronto, Ontario M5S 3H6, Canada*
*Genencor International, Inc., 925 Page Mill Road*
*Palo Alto, California 94304-1013*

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Enzymes are now widely accepted as useful catalysts in organic synthesis.[1] However, natural, wild-type, enzymes can never hope to accept all structures of synthetic chemical interest, nor always to transform them stereospecifically into the desired enantiomerically pure materials needed for synthesis. This potential limitation on the synthetic applicabilities of enzymes has been recognized, and progress has been made towards altering their specificities in a controlled manner using the site-directed and random mutagenesis techniques of protein engineering.[2] However, modifying enzyme properties by protein engineering is limited to making natural amino acid replacements, and molecular biological methods recently devised[3] to overcome this restriction are not readily amenable to routine application or large-scale synthesis. Recently, we have begun to address these issues by combining site-directed mutagenesis with chemical modification.[4] The generation of new specificities or activities obtained by chemical modification of enzymes has intrigued chemists for many years[5] and continues to do so.[6] We have adopted the combined site-directed mutagenesis−chemical modification strategy,[7] since it offers virtually unlimited possibilities for creating new structural environments at any enzyme location. Cysteine is introduced at preselected positions and then reacted with methanethiosulfonate reagents, which react specifically and quantitatively with thiols and are routinely used for chemical modification of proteins.[8] The enzyme chosen was subtilisin *Bacillus lentus* (SBL).[9] This is an ideal template since wild-type SBL itself contains no cysteine. Using the X-ray structure of SBL as our guide,[10] the N62 and L217 residues, of the $S_2$ and $S_1'$ pockets,[11] respectively, were selected for mutagenesis and their cysteine mutants prepared as previously described.[4a]

All too often, site-directed mutagenesis creates mutant enzymes with lower-than-WT activities. We therefore set ourselves the goal of creating chemically modified mutant enzymes (CMMs) that could at least match the activity of the WT parent. With this objective in mind, we reacted SBL-N62C and -L217C (Scheme 1) with the methanethiosulfonates **1a**−**k**,[12] as described previously.[b] The CMMs were fully characterized, and their chemical integrities were established.

The kinetic data obtained, with suc-AAPF-pNA as the reference substrate, are summarized in Table 1. Excitingly, despite the breadth of the structural range of the R groups introduced, virtually all of the chemical modifications resulted in CMMs with higher $k_{cat}/K_M$ values than their cysteine mutant enzyme predecessor, with L217C-**k** being the single exception to this pattern. However, for either site, introductions of the charged sulfonatoethyl (R = **j**) and aminoethyl (R = **k**) groups are clearly only marginally beneficial relative to those of any of the hydrophobic moieties of **1a**−**i**. It is evident that the more hydrophobic the introduced modifying group, the higher the $k_{cat}$ values become. Interestingly, the $K_M$ values for all CMMs of Table 1 are very similar and do not differ significantly from that of the SBL-WT, whereas the $K_M$ values for the SBL-N62C and -L217C mutants themselves are almost 3- and 2-fold higher, respectively, than those for SBL-WT.

The most dramatic effects are manifest for the N62C CMM enzymes. Upon modification with **1a**−**f**, $k_{cat}/K_M$ increases monotonically as the length of the R side chain grows. The greater importance of chain length over steric volume in this regard is demonstrated by the higher $k_{cat}/K_M$ benefit conferred by *n*-pentyl (N62C-**e**) rather than *neo*-pentyl (N62C-**d**) modification. For acyclic alkyl modifications, the maximum activity augmentation is seen for N62C-**f** , for which R = *n*-hexyl and whose $k_{cat}/K_M$ is 6-fold higher than for N62C itself. The kinetic parameters deteriorate slightly with an *n*-decyl group (N62C-**g**), perhaps indicating a steric limitation to the ability of the N62 site to accommodate an introduced substituent. The largest catalytic improvement was observed for the cyclohexylmethyl mutant N62C-**h**, for which the $k_{cat}/K_M$ was almost 7-fold higher than that of its N62C parent. Benzylation was also highly beneficial, with $k_{cat}/K_M$ for N62C-**i** being 4.6-fold elevated. The activity patterns elicited by chemical modifications of L217C are similar. However, now the highest $k_{cat}/K_M$, at 7.8-fold better than that of L217C itself, is reached with the *n*-decyl-modified L217C-**g**, with the cyclohexyl and benzyl modifications of L217C-**h** and -**i**, respectively, being much less advantageous and indeed little better than methylation (L217C-**a**).

While both the lower $k_{cat}$ and increased $K_M$ values exhibited by N62C and L217C relative to SBL-WT are evident in Table 1, the overall effects of chemical modification are more

† Department of Chemistry, University of Toronto.
‡ Department of Molecular and Medical Genetics, University of Toronto.
§ Genencor International, Inc.

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(12) Compounds **1a**, **1j**, and **1k** are commercially available (Toronto Research Chemicals, 4481 Chesswood Dr., Downsview, Ontario M3J 2C3, Canada), whereas compounds **1b**−**1i** were synthesized by substitution reactions of sodium methanethiosulfonate and the corresponding halides.

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**Table 1.** Kinetic Constants for Unmodified and Chemically Modified SBL-N62C and -L217C[a]

| | N62C-R | | | L217C-R | | |
|---|---|---|---|---|---|---|
| R | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) |
| H | 61 ± 2 | 1.49 ± 0.1 | 41 ± 3 | 16.1 ± 0.5 | 0.90 ± 0.08 | 18 ± 2 |
| **a** | 45.5 ± 0.4 | 0.69 ± 0.02 | 66 ± 2 | 27.8 ± 0.9 | 0.57 ± 0.05 | 49 ± 5 |
| **b** | 66 ± 2 | 0.77 ± 0.05 | 86 ± 6 | 31.2 ± 0.9 | 0.53 ± 0.04 | 59 ± 5 |
| **c** | 76 ± 3 | 0.84 ± 0.09 | 90 ± 10 | 37 ± 1 | 0.53 ± 0.05 | 70 ± 7 |
| **d** | 96 ± 2 | 0.78 ± 0.04 | 123 ± 6 | 40 ± 1 | 0.47 ± 0.04 | 85 ± 7 |
| **e** | 138 ± 4 | 0.81 ± 0.06 | 170 ± 13 | 41 ± 1 | 0.42 ± 0.05 | 98 ± 12 |
| **f** | 136 ± 6 | 0.54 ± 0.06 | 252 ± 30 | 61 ± 1 | 0.45 ± 0.03 | 136 ± 9 |
| **g** | 69 ± 3 | 0.35 ± 0.06 | 197 ± 35 | 77 ± 2 | 0.55 ± 0.04 | 140 ± 11 |
| **h** | 135 ± 8 | 0.48 ± 0.09 | 281 ± 55 | 29.8 ± 0.9 | 0.51 ± 0.05 | 58 ± 6 |
| **i** | 70 ± 2 | 0.37 ± 0.03 | 189 ± 16 | 31.6 ± 0.7 | 0.65 ± 0.03 | 49 ± 3 |
| **j** | 66 ± 2 | 0.83 ± 0.05 | 80 ± 5 | 20.6 ± 0.5 | 0.77 ± 0.05 | 27 ± 2 |
| **k** | 63 ± 3 | 1.2 ± 0.1 | 52 ± 5 | 12.4 ± 0.5 | 0.71 ± 0.08 | 17 ± 2 |

[a] Kinetic constants were obtained by nonlinear regression of initial rates determined at eight substrate concentrations (0.25 mM−4.0 mM, suc-AAPF-pNA) at 25 °C in 0.1 M phosphate buffer, pH 7.5, containing 0.5 M NaCl, 1% DMSO. SBL-WT:[4a] $k_{cat}$ = 48 $s^{-1}$; $K_M$ = 0.55 mM; $k_{cat}/K_M$ = 87 $s^{-1}$ $mM^{-1}$.

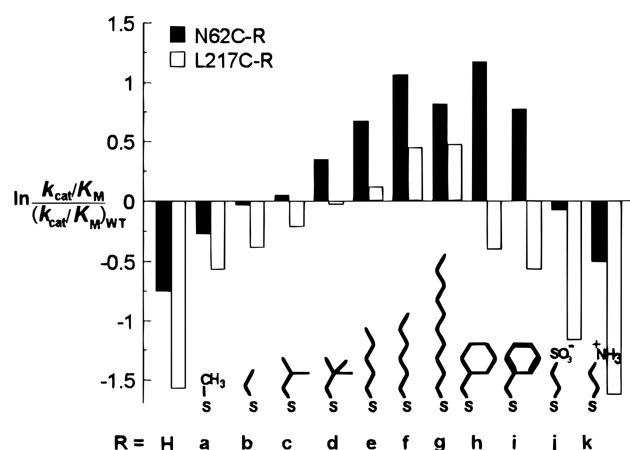
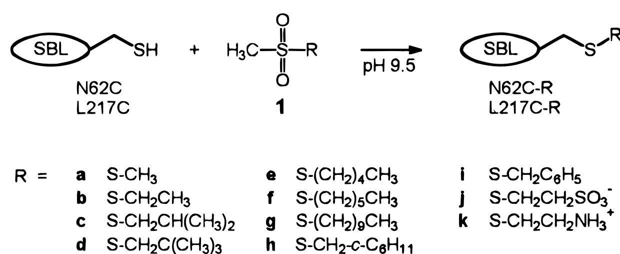



**Figure 1.** Comparison of the $k_{cat}/K_M$ values for the N62C-R and L217C-R enzymes with that of SBL-WT for suc-AAPF-pNA as substrate.

**Scheme 1**




effectively displayed by the $k_{cat}/K_M$ ratios shown in Figure 1. This plot clearly reveals the divergent effects of the various chemical modifications, first to ameliorate the loss of activity engendered by the initial introduction of cysteine by site directed mutagenesis (R = **a**−**c**) and then to surpass (R = **d**−**i**) the WT levels by up to 3.2-fold (R = **h**). The N62C-modified enzymes are more consistently activated than their L217C-analogs, with large, hydrophobic, alkyl groups conferring the most favorable effects. It is remarkable that of only 22 CMMs evaluated, almost half of them not only match the wild-type $k_{cat}/K_M$ level but in fact exceed it by factors of up to >3. To our knowledge, the attainment of these levels of augmented-WT activity for such a broad range of modified enzymes is unprecedented and has not been matched so far in its breadth by protein engineering methods alone. The deleterious, relative to WT, effects of both negatively and positively charged modifications (**j**, **k**) are also evident from this plot.

These results demonstrate the considerable potential that the combined site-directed mutagenesis−chemical modification approach offers for creating novel enzymes with better-than-WT activity. The opportunities for generating catalysts with other improved properties, such as changed specificities and stabilities, are self-evident and are being explored. It is interesting to note that chemical modification effects can mimic those effected by traditional protein engineering. For example, the $k_{cat}$ enhancements elicited by chemical modification of SBL-L217C with hydrophobic side chains (**a**−**i**) and the modification of SBL-N62C with the polar side chain **j** mirror those induced by introduction of amino acids with analogous hydrophobic (methionine and leucine) or charged (aspartic acid) amino acid side chains by site-directed mutagenesis at the 217 and 62 positions, respectively, in the closely related enzyme subtilisin BPN′.[13,14] Thus, a further benefit of the current approach may be to provide a rapid and convenient screen identifying the most desirable types of amino acid replacements for a given enzyme location by traditional protein engineering methodology.

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**Supporting Information Available:** Chemical modification procedures, characterization of CMMs, and preparation of methanethiosulfonate reagents (20 pages). See any current masthead page for ordering and Internet access instructions.

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