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Fluorinated chloramphenicol acetyltransferase thermostability and activity profile: Improved thermostability by a single-isoleucine mutant

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Abstract—A lysate-based thermostability and activity profile is described for chloramphenicol acetyltransferase (CAT) expressed in trifluoroleucine, T (CAT T). CAT and 13 single-isoleucine CAT mutants were expressed in medium supplemented with T and assayed for thermostability on cell lysates. Although fluorinated mutants, L82I T and L208I T, showed losses in thermostability, the L158I T fluorinated mutant demonstrated an enhanced thermostability relative to CAT T. Further characterization of L158I T suggested that T at position 158 contributed to a portion of the observed loss in thermostability upon global fluorination.
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Non-natural amino acids have been used to create defined mutations that are unique from the set of amino acid monomers in naturally occurring proteins. Introduction of a tailored side chain at one or more residues has been used to explore the effects of particular hydrogen bonds on protein folding,[1] to stabilize protein conformations,[2] and to probe the catalytic mechanisms of enzymes.[3] Residue-specific incorporation of non-natural amino acids allows for the global replacement of a natural amino acid in vivo.[4,5] This form of multi-site substitution can be used to alter the overall properties of proteins.[6,7] Substitution of leucine with trifluoroleucine (T) or hexafluoroleucine (H) in a coiled-coil protein revealed an enhanced stability toward thermal and chemical denaturation.[8–11] By contrast, the residue-specific incorporation of T into chloramphenicol acetyltransferase (CAT) resulted in a loss of thermostability.[12]

To deconvolute those fluorinated residues that contribute to the observed decrease in thermostability of the fluorinated CAT (CAT T), we merged site-directed mutagenesis with residue-specific incorporation in which each T was mutated into isoleucine. CAT and 13 single-isoleucine CAT mutants were expressed in medium supplemented with T and assayed for thermostability on cell lysates.[13] This was done to prevent the introduction of T at specified positions. Here, we report the thermostability profile of wild-type CAT and 13 single-isoleucine CAT mutants expressed in the presence of T (and leucine, L, as a control) by utilizing a microplate-based lysate activity screen.

The development of a robust screen that can be easily monitored is critical for the identification of mutants bearing enhanced or altered properties.[14] We measured the activity of *Escherichia coli* cell lysates from a 5 mL growth at 412 nm in the presence of chloramphenicol, acetyl coenzyme A, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to measure the activity of crude protein (Fig. 1a). For cells bearing CAT (positive control), the lysates exhibited an increase in intensity at 412 nm, which indicated catalytic activity. Cell lysates in the absence of CAT (negative control) did not demonstrate any increase in intensity above background.

Single colonies bearing the CAT gene were grown and expressed in microtiter plates to adapt the assay for

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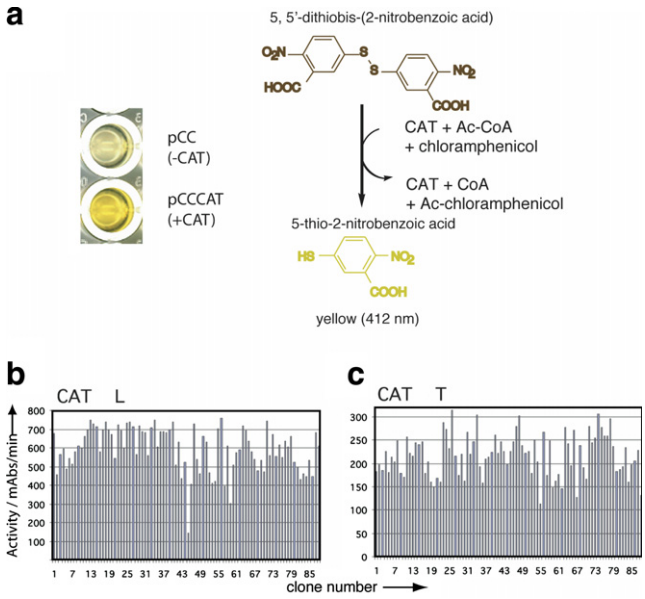

Figure 1. (a) Activity assay on cell lysates. In the absence of the CAT gene there is no observed color change above background, indicating no activity, while in the presence of the CAT gene there is a distinct color change upon formation of 5-thio-2-nitrobenzoic acid (TNB) that can be monitored at 412 nm. Validation of CAT cell lysates grown in the presence of (b) 20 natural amino acids and (c) 19 amino acids supplemented with T.

96-well format. The variability of the activities of a single sequence CAT library was assessed to determine the reproducibility of the screen.[14] The coefficient of variance for the 96-well plate bearing CAT expressed in medium supplemented with the natural set of 20 amino acids (CAT L) was calculated as 19.3% (Fig. 1b). Another single sequence CAT library was expressed in medium supplemented with T (CAT T). A medium shift was performed on the 96-well plate, where the wells were washed twice with 0.9% NaCl before the addition of the new medium bearing T to enhance the incorporation of the non-natural amino acid. The coefficient of variance was 23.2% in the presence of T (Fig. 1c). To evaluate the extent of T incorporation in the wells, all the lysates were pooled, purified, and determined to possess 63% substitution by amino acid analysis. Prior work had demonstrated that the incorporation level leads to different extent of stability; low levels such as 17% or 37% T substitution exhibited distinct half-lives at 60 °C.[12] Although this was lower than the amount of incorporation obtained for large-scale expression previously[12] and below, comparisons were made under the same degree of substitution.

To identify fluorinated mutants that differ in thermostability (enhanced or diminished) from CAT T, the screen must be sensitive enough to observe small changes in thermostability that result from a single amino acid substitution.[15] Here, thermostability is a measure of the residual activity (RA) after incubation at elevated temperatures normalized to the activity at ambient temperatures.[16,17] Thirteen single-leucine to isoleucine mutants of CAT were generated to investigate the contribution of each T toward the thermostability of CAT T as defined above. We reasoned that T (or L) to isoleucine mutation would preserve the side-chain hydrophobicity and surface area,[18] creating minimal perturbation to the structure and function of CAT T. By making such a homologous substitution, we were able to probe the effect of each T within the context of the fluorinated CAT T. Although a single substitution approach would not rule out the synergistic effects of multiple T interactions, it would provide information as to which of the fluorinated residues is critical to its function.

In a 96-well microtiter plate, six replicates of each mutant were grown and expressed in 19 amino acids supplemented with T and with L as a control. The lysate activity after exposure to elevated temperatures or RA of CAT T and single-isoleucine mutants was measured (Fig. 2a). The lysate activity for each enzyme was also assessed under ambient temperatures to determine whether the differences in thermostability were due to losses or improvements of initial activity (Fig. 2b). Thermostability and activity of mutants expressed in medium supplemented with T (fluorinated conditions) or with L (conventional conditions) were normalized to CAT T or CAT L, respectively. Ten of the 13 mutants showed similar thermostabilities and initial activities when directly compared to fluorinated or conventional CAT protein (Fig. 2a). Most of the T and L residues were permissive to an isoleucine substitution, indicating that each of these T or L residues does not contribute to thermostability or activity in either the fluorinated or conventional contexts.

Two fluorinated mutants, L82I T and L208I T, demonstrated a loss in thermostability relative to CAT T, suggesting that a mutation into isoleucine at those positions destabilized the enzyme (Fig. 2a). The activities at room temperature of both mutants were compared to CAT T to investigate whether the observed reduction in thermostability was due to loss in initial activity. Fluorinated mutant L82I T revealed a similar initial activity relative to CAT T (Fig. 2b). In the case of L208I T, substitution with isoleucine negatively affected the initial activity with respect to CAT T, indicating that isoleucine at position 208 possesses a deleterious effect on enzyme function (Fig. 2b). The same trend was observed under conventional 20 amino acid conditions.

Remarkably, fluorinated mutant L158I T revealed a 1.8-fold increase in thermostability relative to CAT T (Fig. 2a). The same mutation expressed in medium bearing 20 amino acids, L158I L, exhibited similar thermostability relative to CAT L. Thus, the isoleucine mutant only exhibited an effect in the context of the fluorinated protein. The observed improvement in thermostability of L158I T was not a result of initial activity changes; L158I in conventional and fluorinated contexts exhibited comparable initial activities to their wild-type counterparts.

To quantify the enhancement in thermostability, mutant L158I was expressed in medium supplemented with T or L, purified, and characterized. The activity and thermostability of the mutants were compared to CAT T and CAT L, respectively. For L158I T and CAT T, mass

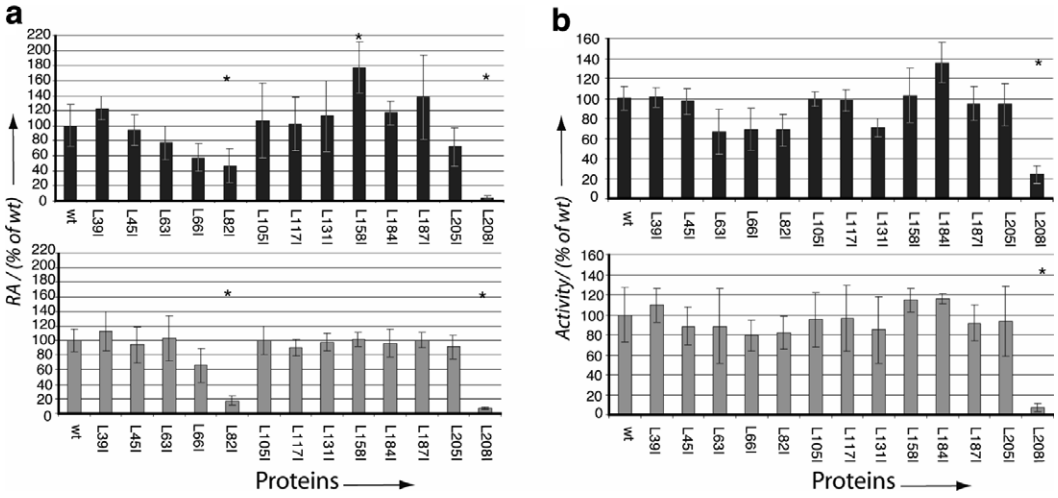

Figure 2. (a) Plot of the RA of CAT and isoleucine mutants. (b) Plot of the activity of CAT and isoleucine mutants at room temperature. Gray bars signify enzymes expressed in 20 amino acids while black bars signify enzymes expressed in 19 amino acids supplemented with T. Asterisks highlight thermostability or activity differences that are statistically different from wild-type CAT. Data represent an average of six trials. Error bars denote standard deviation.

spectrometry and amino acid analysis demonstrated an 82% and 83% T substitution, respectively (Supporting information). Quantitative levels of incorporation were not achieved as a result of the trace amounts of natural leucine liberated by degradation of cellular proteins.[12] The 82–83% T incorporation was sufficiently high to avoid the possibility for the synthesis of wild-type protein that could account for the improved thermostability observed. Furthermore, since the comparison of the fluorinated proteins is made on proteins with nearly identical levels of T incorporation, the enhancements observed can be attributed to the protein. The specific activities of CAT and L158I reveal that the catalytic efficiencies were essentially unchanged under both fluorinated and conventional conditions (Table 1). A 2.4-fold increase in K_m was observed for L158I mutant when compared to the CAT bearing T or L. Since the increase in K_m was accompanied by a concomitant increase in k_{cat}, the overall values of k_{cat}/K_m for the conventional and fluorinated proteins were similar, indicating that activity was not altered drastically by the isoleucine substitution and upon fluorination.

The thermostability data on the purified proteins as measured by RA[17] were highly consistent when compared to the data obtained from the lysate assay. Under fluorinated conditions, L158I T demonstrated a 1.5-fold increase in RA relative to CAT T (Table 1 and Fig. 3, Supporting information). In the context of the 20 natural amino acids, L158I L demonstrated a slight decrease in RA relative to CAT L (Table 1 and Fig. 3, Supporting information).

Table 1. Kinetic parameters and thermostability

Protein	K_m (μM)	k_{cat}/K_m (10^4 μM^{-1} min^{-1})	RA[a] (%)
CAT L	13.3 ± 2.0	6.0 ± 0.9	94.9
CAT T	11.8 ± 0.7	5.5 ± 0.3	34.6
L158I L	32.2 ± 2.9	4.5 ± 0.4	89.0
L158I T	28.3 ± 4.9	5.0 ± 0.9	51.7

Kinetic measurements are the average of three experiments (±standard deviation).

[a] Residual activity at 55 °C. Values of RAs are calculated from an average of three experiments (standard deviation <5%).

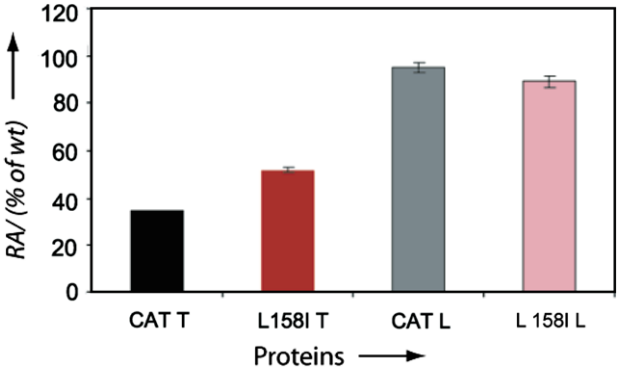

Figure 3. Plots of RA of purified proteins. CAT T is black, L158I T is red, CAT L is gray and L158I L is pink. Data represent an average of three trials. Error bars denote standard deviation.

By modeling the crystal structure of CAT-I with that of the CAT-III chloramphenicol[19,20] complex, we can identify the isoleucine mutants and their positions relative to chloramphenicol (Fig. 4). First, we consider the two mutants that resulted in reduced thermostability: L82I T and L208I T. Structural analysis reveals that both mutations point away from the chloramphenicol-binding pocket and interact with other hydrophobic residues. L82 is positioned on sheet D and is stabilized by a set of hydrophobic residues presented by V11, W16, M75, and M77. L208 is located on helix 5 and is stabilized by all the interior leucines, L39, L63, L66, L187, and L205 or in the case of the fluorinated enzyme, interior TFLs (Fig. 4). The interactions of these residues may be significant in maintaining the proper packing of the CAT structure critical for catalysis. Previous work

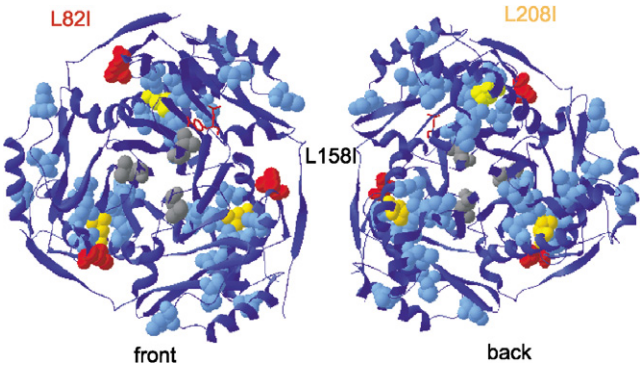

Figure 4. Model of *E. coli* CAT-I trimer bound to chloramphenicol (red). Two of the mutations that result in thermostability loss, L82I and L208I, are depicted in yellow and red, respectively. The mutation that leads to an enhancement in thermostability, L158I, is represented in gray. Residues highlighted in blue are the leucine/TFL residues of CAT that when mutated into isoleucine does not significantly alter the thermostability or activity.

points to the importance of the C-terminal helix for CAT activity.[21] Deletion of 9 residues within helix 5 of CAT-I (CATΔ9) resulted in an inactivate enzyme due to misfolding or structural destabilization.[21,22] Although isoleucine presented similar hydrophobic interactions with leucine or TFL, the difference in the configuration of the side chains could create destabilizing contacts, leading to the observed decrease in thermostability.

Next, we consider L158I T, which resulted in an enhanced themostability. According to our model, L158 is positioned approximately 4 Å from the substrate (Fig. 4). The leucine is located on the outer region on sheet H and is stabilized by the predominantly hydrophobic interactions with N34, V160, F166, and V170 (Fig. 4). Substitution of TFL at 158 with isoleucine improves the thermostability of the fluorinated enzyme, suggesting that the TFL contributes negatively to thermostability. Because it is located on the outside and faces away from the core, the presence of TFL at this position may result in misfolding, leading to instability.[12] Here, isoleucine provides a compensatory mutation that allows for the recovery of a portion of the thermostability that is lost upon global fluorination.

These results confirm that a mutation under two compositionally different proteins leads to different outcomes. Moreover, this demonstrates that a single isoleucine mutation can improve the properties of the fluorinated protein. Since the isoleucine mutation in the context of fluorination led to an enhanced thermostability, the data suggest that the observed loss in thermostability of CAT T was due in part by T at position 158. The T residue at 158 appears to be critical to thermostability and a simple substitution to isoleucine can improve the thermotolerance of the fluorinated protein, possibly by the prevention of misfolding.[12] In order to truly understand the mode by which this substitution is improving the thermostability, three-dimensional structural studies are needed.

Previously, functional proteins bearing non-natural amino acids have been limited to the appropriate placement of the analog,[2,8–11] with some recent exceptions.[23,24] By using this approach, we can now gain insight into the effects of the non-natural amino acid on the protein function and pinpoint which non-natural residues are critical to function. Because the screen developed here provides a functional profile of each non-natural amino acid side chain, it can be used to improve the understanding of non-natural proteins even when the structures of such proteins are not yet determined.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.07.107.

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