An Efficient and Economical Assay to Screen for Triclosan Binding to FabI

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Short Title: Screening Triclosan Binding to FabI

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Abstract

Triclosan is an effective inhibitor for enoyl acyl carrier protein reductase (ENR) in fatty acid biosynthesis. Triclosan-resistant mutants of ENR have emerged. Thus, it is important to detect these triclosan-resistant mutations in ENR. Generally, enzyme activity assays on the mutants are used to determine the effect of triclosan on ENR activity. Since the substrates are linked to acyl carrier protein (ACP), the assays are challenging due to the need to prepare the ACP and link it to the substrates. Though non-ACP linked (CoA-linked) substrates can be used in some ENR, but not in all. Consequently screening for triclosan-resistant mutants are also challenging. We have developed a simple thermal shift assay, which does not use ACP-linked substrates, to determine the binding ability of triclosan to ENR active site, and thus can be used for screening for triclosan-resistant mutants. Staphylococcus aureus FabI enzyme and its mutants were used to demonstrate the binding ability of triclosan with NADP+ to FabI. The direct correlation between the binding ability and enzyme activity was demonstrated with Francisella tularensis FabI. This method may also be applied to select effective triclosan analogues that inhibit ENR activity.

Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy)phenol, or 2,4,4'-trichloro-2'-hydroxydiphenyl
ether, has activity against many, but not all, types of Gram-positive and Gram-negative bacteria\textsuperscript{1,2,3}, including methicillin resistant Gram-positive \textit{Staphylococcus aureus} (\textit{S. aureus}) strains\textsuperscript{4}, \textit{Mycobacterium tuberculosis}\textsuperscript{5,6}, \textit{Plasmodium falciparum}\textsuperscript{7}, etc. It has also been reported that triclosan is cytotoxic to breast cancer cells\textsuperscript{8}. The mode of action of triclosan on bacterial cells is well studied\textsuperscript{3,9,10,11,12}, and it targets enoyl-acyl carrier protein (ACP) reductase (ENR), which is encoded by the gene \textit{FabI}\textsuperscript{11} and catalyzes the final step of the type II (bacterial) fatty acid biosynthesis pathway to reduce the double bond of enoyl-ACP to single bond acyl-ACP with cofactor NAD(P)H\textsuperscript{13}. Triclosan inhibits the enzyme action by forming a stable ternary complex with the oxidized cofactor NAD(P)+ and the side chains of FabI active site residues\textsuperscript{13}. The triclosan effect on human fatty acid synthase study suggest that inhibitors like triclosan may have chemotherapeutic potential\textsuperscript{8}.

Due to its usefulness toward inhibiting bacterial growth, triclosan has been widely used\textsuperscript{14}, and species resistant to triclosan can arise from mutations in ENR/FabI. For example, \textit{S. aureus} FabI (SaFabI) mutations such as A95V, I193S, and F204S have been identified in selection experiments\textsuperscript{15}, suggesting that other mutations in \textit{S. aureus} may also survive triclosan treatment, and need to be identified. To determine whether triclosan inhibits certain ENR/FabI mutants, enzymatic activity in the presence of triclosan is commonly measured to determine triclosan inhibition activity. For ENR/FabI enzymes of many species such as \textit{Escherichia coli} (\textit{E. coli}) and \textit{Francisella tularensis} (\textit{F. tularensis}), it is possible to substitute the substrate enoyl-ACP with CoA esters, such as crotonoyl-CoA, as experimental substrates\textsuperscript{16,17}, but with much reduced activities\textsuperscript{17}. For the ENR/FabI of species where the substrate cannot be substituted with crotonoyl-CoA, such as that of \textit{S. Aureus}, substrate enoyl-ACP need to be used to identify triclosan-resistant mutations, or to select effective triclosan analogs as useful antibiotic leads. Obtaining ACP-linked substrates involves the purification of apo-ACP from cellular extract followed by either enzymatic-driven\textsuperscript{15,18}, or chemically-driven\textsuperscript{19} linkage of the enoyl chain to the ACP. Consequently, studies that require enoyl-ACP for substrate are laborious. It is speculated
that SaFabI diverges from the classical FabI structure and behavior owing to the wealth of branched chain fatty acids occupying the membranes of *S. aureus*\(^{20}\). If so, the traits of SaFabI may be repeated in other organisms with similar membrane compositions like the genus *Bacillus*, in which all of the species are with branched fatty acid chains in their membranes\(^{20,21}\).

We have developed a thermal shift method to determine whether triclosan binds specifically to the active site of FabI without the use of enoyl-ACP. This thermal shift assay is a sensitive fluorescence-based assay\(^{22}\), making it amenable to high-throughput screening. We showed that triclosan, together with NADP\(^+\), binds to SaFabI wild-type and triclosan-sensitive mutant M99T, but not to triclosan-resistant mutants A95V, I193S, and F204S. We used the *F. tularensis* FabI (*FtFabI*) system, wild-type and a triclosan-resistant mutant, to demonstrate the direct correlation between their thermal shifts and enzyme activities. We also show that the thermal shift is not due to the fluorescent probe used in the assay. We demonstrate that this simple and economical method, without using enoyl-ACP substrate, can be used to screen for triclosan-resistant mutants of ENR/FabI.

### Method and Materials

#### Recombinant Plasmids

The SaFabI gene (NWMN_0881) was amplified from *S. aureus* gDNA and inserted in a pET-15b vector to express recombinant His-tagged SaFabI wild type (WT). AFN-1252 resistant, but triclosan-sensitive M99T mutation\(^{23}\), and triclosan-resistant mutations A95V, I193S, and F204S mutants\(^{15}\) were constructed with site-directed primer mutagenesis method\(^{24}\). The *FtFabI* gene (FTT_0782) was amplified from *F. tularensis* gDNA and inserted in a pET-15b vector to express recombinant His-tagged *FtFabI* WT. The A92V mutation, a homologous mutation to *E. coli* FabI (*EcFabI*) G93V triclosan-resistant mutation\(^{10}\), was also constructed using the same method. All resulting gene constructs were sequenced at the University of Illinois at Chicago Research Resource Center (UIC RRC).
Protein Expression and Purification

*E. coli* competent cells (BL21 CodonPlus(DE3)-RII; Zymo) was used to express both WT and mutant proteins of both *S. aureus* and *F. tularensis*. An overnight culture (100 mL) was added to fresh Lysogeny Broth medium (1 L in a 2.8-liter culture flask). Cells were grown to an optical density at 600 nm of 0.5 - 0.8 at 37 °C with shaking. Isopropyl β-D-1-thiogalactopyranoside was then added to a final concentration of 0.5 - 1.0 mM to induce protein expression. Cells were grown for an additional 3 h after induction, and harvested at 4 °C.

The cells in 50 mM sodium phosphate buffer at pH 8 with 300 mM NaCl (phosphate buffer) plus 10 mM imidazole and 1% Triton X-100 were sonicated for 20 min on ice slurry. The mixture was centrifuged at 35,000 g for 30 min to give cell lysate. The lysate was loaded to a nickel affinity column (Qiagen) pre-equilibrated with the phosphate buffer plus 10 mM imidazole. The His-tagged protein was released from the column with the phosphate buffer plus 300 mM imidazole and dialyzed in 50 mM Tris buffer at pH 8 with 100 mM NaCl (Tris buffer) before concentrating to ~100 - 300 uM. The protein samples were frozen drop-wise (20 uL) in liquid nitrogen and stored at -80 °C until needed. The protein purity was monitored with 16% polyacrylamide gel electrophoresis, and the molecular mass of the proteins was checked with high resolution mass spectrometry at UIC RRC.

The concentration of each protein was determined from the absorbance at 280 nm and the extinction coefficient derived from its sequence, with 13,410 M⁻¹cm⁻¹ for *SaFabI* WT, A95V, M99T, I193S, and F204S, and 17,420 M⁻¹cm⁻¹ for *FtFabI* WT and A92V.

Enzymatic Assay for *FtFabI*

The published method for the enzymatic activity of *EcFabI* WT using crotonyl-CoA as substrate was adapted for that of *FtFabI*. Briefly, *FtFabI* WT (450 nM) or A92V (450 nM and 1 uM) in Tris buffer were incubated with 250 uM NADH and 200 uM NAD⁺, without and with triclosan (2.5 molar ratio to FabI; triclosan from Sigma, 72779-5G-F) for 5 h at 4 °C. Since triclosan was in dimethyl sulfoxide (DMSO), both samples also consisted of 1% (v/v) DMSO.
The relatively long incubation time (5 h) was to be consistent with the condition used for SaFabl in the thermal shift assay to ensure the complex formation between SaFabl, NADP⁺, and triclosan. It is known that triclosan exhibits its inhibition activity by forming a tight complex with Fabl and NAD(P)⁺, and it is also known that NAD(P)⁺ itself exhibits relatively low affinity toward Fabl and is in competition for the binding site¹²,²⁰. At the end of the incubation, the mixtures were warmed to 25 °C (in about 10 min) followed by the addition of an experimental substrate, crotonyl-CoA (200 uM final concentration; Sigma, C6146-5MG) to start the reaction. The decrease in NADH concentration, upon oxidation to NAD⁺ during the catalytic reaction, was monitored by UV absorption at 340 nm (A₃₄₀) for ~2.5 min with a plate reader (Victor³ V; Perkin Elmer). The slope of a linear fit of A₃₄₀ versus time plot (ΔA₃₄₀/Δmin) was converted to a rate of NADH consumed, using the NADH extinction coefficient of 6220 M⁻¹ cm⁻¹. Specific activity for FlFabl was calculated as the rate of NADH consumed (umol/min) per mg of FlFabl.

**Thermal Shift Assay for Binding Triclosan**

Samples consisting of SaFabl (7 uM in Tris buffer) and 5x Sypro Orange (Invitrogen) alone (control), or with only triclosan (100 uM) (sample-T), with only NADP⁺ (200 uM) (sample-N), or with both triclosan and NADP⁺ (sample-TN) were prepared side-by-side, with the same batch of protein, triclosan, and Sypro Orange to reduce sample variability. The control and the 3 samples (sample-T, sample-N, and sample TN) also included 1% (v/v) DMSO since both Sypro Orange and triclosan stock solutions were in DMSO. Sypro Orange was supplied in DMSO as 5,000x stock solution. The mixtures were incubated for 5 h at 4 °C, as in the activity studies. Some samples were incubated for only 30 min. In the FlFabl samples, instead of NADP⁺, NAD⁺ was used.

The fluorescence intensity of Sypro Orange at 570 nm with excitation at 472 nm was monitored with a FP-6200 Jasco Spectrofluorometer as a function of temperature in the range of 25 - 75 °C. The temperature ramp rate was 1.0 °C/min. The intensity values were converted to fraction unfolded.
A Boltzmann sigmoidal fit, modeling a two-state unfolding process, was applied to the data to give transition temperatures measured by Sypro Orange ($T_m^{SO}$). A difference in the transition temperatures (thermal shift, $\Delta T_m^{SO}$) between control and samples was obtained, and the $\Delta T_m^{SO}$ between control and sample-TN was used to indicate a degree of stabilization of FabI upon binding triclosan and NAD(P)$^+$ to the active site of FabI.

**Effects of Sypro Orange on Thermal Unfolding**

The circular dichroism (CD) ellipticity ($\theta$) signals at 222 nm (with a Jasco J-810 Spectropolarimeter) of *Ft* FabI and *Sa* FabI (15 uM) in the Tris buffer were measured from 25 - 75 °C with a ramp rate of 1.0 °C/min. $\theta$ values were converted to fractions of unfolding, with $\theta$ at 25 °C as 0 and $\theta$ at 75 °C as 1. Again a Boltzmann fit was applied to the data to give transition temperatures measured by CD ($T_m^{CD}$). Also measured were FabI samples with 5x Sypro Orange and/or 0.1% (v/v) DMSO. We were not able to do CD measurements in the presence of 1% DMSO (as in activity assay and thermal shift assay) due to the strong CD signal from 1% DMSO. For the same reason, CD method was not used to monitor thermal shifts of samples with and without triclosan and NAD(P)$^+$, since the samples included 1% DMSO.

**Results**

**Enzyme Solution Properties**

The polyacrylamide gel showed a band around 30 kDa for each of the seven over-expressed proteins, with 80 - 90% purity. High resolution mass spectrometry results indicated that the initial methionine in the His-tag was missing in all our proteins, and the masses were within 1 Da of the expected values derived from sequences. For example, for *Sa* FabI WT, the difference between the sequence-derived mass (30397.4 Da) and experimental mass (30396.7 Da) was -0.7 Da. For *Sa* FabI M99T, A95V, I193S, and F204S, the sequence-derived masses were 30367.3 Da, 30425.3 Da, 30371.3 Da, and 30337.3 Da, respectively, and the experimental masses were 30366.6 Da, 30424.8 Da, 30370.6 Da, and 30336.6 Da, respectively.

*Ft* FabI WT enzyme exhibited a specific activity of 0.66 umol min$^{-1}$ mg$^{-1}$ (Fig 1). In the
presence of triclosan, the specific activity decreased to 0.06 umol/min⁻¹ mg⁻¹, with only 9% activity remaining, clearly demonstrating the inhibitory effect of triclosan on FtFabI. For the A92V mutant at the concentration used for WT measurements (450 nM), the decrease of A₃₄₀ was small, making it difficult to obtain an accurate value for inhibition (Fig 1). However, with the enzyme concentration increased to 1 uM, the decrease in A₃₄₀ as a function of time was more pronounced, and the normalized specific activity value for A92V was 0.09 umol min⁻¹ mg⁻¹. In the presence of triclosan, the specific activity was measured as 0.10 umol min⁻¹ mg⁻¹. Thus, A92V, with much reduced activity as compared to that of the WT, was not inhibited by triclosan.

The enzyme activity for SaFabI was not measured, as it requires enoyl-ACP as the substrate, as mentioned above.

**Thermal Shifts for Triclosan Binding to Active Site**

**SaFabI**

The TₘSO average value from thermal unfolding profiles of SaFabI WT (Fig 2) was 40.5 ± 1.0 °C (n = 6). With the addition of just triclosan alone, the average value was 37.6 ± 0.9 °C (n=2), and of just NADP⁺, the average value was 40.2 ± 1.2 °C (n=2). Neither triclosan nor NADP⁺ increased the TₘSO values. For SaFabI WT with both triclosan and NADP⁺ (Fig 2), the average value was 58.8 ± 1.6 °C (n = 2). A thermal shift of about 20 °C was observed for SaFabI WT upon addition of both triclosan and NADP⁺, but no shift upon addition of just triclosan or NADP⁺. Since the addition of triclosan or NADP⁺ alone did not stabilize SaFabI, and only when both were added was SaFabI stabilized, the results, together with the knowledge that triclosan and NADP⁺ bind as a complex to the active site of SaFabI²⁰, suggest that the observed thermal shift reflects not only binding to SaFabI, but specific binding to the active site of SaFabI.

The TₘSO average value of SaFabI M99T (Fig 2) was 40.2 ± 0.8 °C (n = 3). With the addition of just triclosan alone, the average value was 40.8 ± 0.1 °C (n=2), and of just NADP⁺, the average value was 41.2 ± 0.1 °C (n=2). For SaFabI M99T with triclosan and NAD⁺, the average value was 60.2 ± 1.1 °C (n = 2). An approximate 20 °C thermal shift was observed for
SaFabl M99T upon binding triclosan and NADP⁺. It is interesting to note that 30 min incubation allowed some of the triclosan and NADP⁺ to bind to WT and M99T molecules, but not all, resulting a thermal shift profile of two transitions (Fig 2). The SaFabl WT sample consisted of two populations, one without and one with triclosan and NADP⁺, and the first transition was at 40.9 °C, representing the thermal transition of the WT without triclosan and NADP⁺, and the second transition was at 60.9 °C, representing the thermal transition of WT stabilized by triclosan and NADP⁺. Similarly, for M99T, the triclosan-sensitive mutant, we also found a transition at 39.8 °C and another transition at 61.4 °C (Fig 2).

The $T_m^{SO}$ average value for A95V (Fig 3) without triclosan and NADP⁺ was $39.4 \pm 0.2$ °C ($n = 3$), quite similar to that of the WT (Fig 2), and with triclosan and NADP⁺ was $40.3 \pm 0.1$ °C ($n = 2$). No thermal shift was observed for SaFabl A95V. Similarly, the average value for I193S (Fig 3) without triclosan and NADP⁺ was $40.3 \pm 0.0$ °C ($n = 2$), and with triclosan and NADP⁺ was $40.2 \pm 0.1$ °C ($n = 2$). No thermal shift was observed for SaFabl I193S. The average value for F204S (Fig 3) without triclosan and NADP⁺ was $41.1 \pm 0.1$ °C ($n = 2$), and with triclosan and NADP⁺ was $41.2 \pm 0.1$ °C ($n = 2$). No thermal shift was observed. Our results showed no binding of triclosan and NADP⁺ to SaFabl mutants A95V, I193S and F204S.

FtFabl

The $T_m^{SO}$ average value for FtFabl WT (Fig 4) was $53.2 \pm 0.4$ °C ($n = 4$). With the addition of just triclosan alone, the average $T_m^{SO}$ was $52.3 \pm 0.0$ °C ($n = 2$), and of just NAD⁺ the average $T_m^{SO}$ was $52.4 \pm 0.9$ °C ($n = 2$). For FtFabl WT with triclosan and NAD⁺, the average $T_m^{SO}$ was $60.2 \pm 3.4$ °C ($n = 2$). About 7 °C thermal shift was observed for FtFabl. Similar thermal shift was observed ($60.8 \pm 0.5$ °C; $n = 3$) for samples incubated for 30 minutes. The average value for A92V without triclosan and NAD⁺ was $51.7 \pm 0.5$ °C ($n = 3$), and with triclosan and NAD⁺ was $52.1 \pm 0.1$ °C ($n = 2$). No thermal shift was observed for FtFabl A92V. Together with the enzyme activity values (Fig 1), we showed that, using the FtFabl system, the thermal shifts and the activity decreases upon addition of triclosan and NAD⁺ are well correlated.
Triclosan and NAD$^+$ together stabilized *FtFabI* WT to increase the thermal transition temperature by 7 °C, and inhibited the enzymatic activity by 91%. For *FtFabI* A92V, no thermal shift was observed, and no change in the residual enzymatic activity in the presence of triclosan and NAD$^+$. It was interesting to note that, unlike in the *SaFabI* system, 30 min incubation of triclosan and NAD$^+$ with *FtFabI* was sufficient to fully stabilize *FtFabI* WT (Fig 4).

**Sypro Orange Effect on FabI Stability**

Since we used Sypro Orange to report the thermal unfolding, we investigated whether Sypro Orange in DMSO affected the thermal unfolding by using CD to monitor the unfolding of FabI with and without 5x Sypro Orange in DMSO (0.1%, v/v). Due to large CD signal for DMSO, we used only 0.1% DMSO, rather than 1% as used in the $T_{mSO}$ studies. Enzyme activities with and without 1% DMSO were about the same. *FtFabI* WT without DMSO, with 0.1% DMSO, or with 0.1% DMSO and 5x Sypro Orange revealed similar thermal unfolding profiles of secondary structures (Fig 5). The $T_m^{CD}$ average values for samples without ($n = 3$) and with ($n = 2$) DMSO (0.1%) were both 57.7 ± 0.1 °C, and for samples with 0.1% DMSO and 5x Sypro Orange, the value was 56.4 ± 0.2 ($n = 2$). Similar profiles, except shifted to lower temperatures, were observed for *SaFabI* WT, with $T_m^{CD}$ of 43.7 ± 0.0 °C ($n = 2$). For *SaFabI* WT in 0.1% DMSO, the $T_m^{CD}$ was 43.6 ± 0.0 ($n = 2$), and in 0.1% DMSO and 5x Sypro Orange, the $T_m^{CD}$ was 42.7 ± 0.0 ($n = 2$). Little DMSO or 5x Sypro Orange effect was observed.

The $T_m^{CD}$ and the $T_m^{SO}$ values of the same sample differed slightly since the two methods measured slightly different unfolding events, with the CD reporting the unfolding of the secondary structures and the SO reporting the exposed hydrophobic moieties during unfolding.

**Discussion**

When Sypro Orange is added to intact protein in solution, which has little hydrophobic moieties, it exhibits little fluorescence intensity. However, when protein is thermally unfolded to expose hydrophobic moieties, Sypro Orange molecules around these moieties exhibit fluorescence intensity. By following Sypro Orange fluorescence intensity in a protein sample
as a function of temperature, protein unfolding profiles with characteristic transition temperatures are obtained. Since compounds binding to a protein molecule lower the free energy of the system\textsuperscript{26}, Sypro Orange has been widely used to track the stabilisation of a protein molecule due to compound binding\textsuperscript{22,27,28}.

We showed that we may use this principle to determine whether triclosan and NAD/P\textsuperscript{+} bind to Fabl mutants or not. The characteristic transition temperature in thermal unfolding of SaFabl WT in Tris buffer was about 40 °C. The temperature did not change upon addition of just triclosan or just NADP\textsuperscript{+}. However, SaFabl WT and M99T was stabilized by the triclosan and NADP\textsuperscript{+} with the transition temperature shifted about 20 °C. For known triclosan-resistant mutants, A95V, I193S, and F204S, no thermal shift was observed upon addition of triclosan and NADP\textsuperscript{+}. It should also be noted that all SaFabl samples, WT and the mutants, exhibited similar transition temperatures (~40 °C) in the absence of triclosan and NADP\textsuperscript{+}. Since the thermal shifts were observed only when both triclosan and NADP\textsuperscript{+} were present, but not when only one was present, we suggest that the binding we observed was the active-site binding observed previously\textsuperscript{20}.

To demonstrate that the active-site bindings indicated by thermal shifts are related to enzyme activities, we used the FtFabl system. The transition temperature for FtFabl in Tris buffer was about 53 °C but shifted to 60 °C upon addition of triclosan and NAD\textsuperscript{+}. We also showed that the specific activity for FtFabl was inhibited by triclosan and NAD\textsuperscript{+}. We further showed that a FtFabl triclosan-resistant mutant, A92V, with much reduced specific activity when compared with that of the WT, but was not further reduced upon addition of triclosan and NAD\textsuperscript{+}. This mutant exhibited little thermal shift upon addition of triclosan/NAD\textsuperscript{+} in its thermal unfolding profile.

It was interesting to note that a 30 min incubation period was sufficient to fully stabilize FtFabl WT with triclosan and NAD\textsuperscript{+}. However, the unfolding profiles of SaFabl WT and M99T samples with 30 min incubation clearly showed incomplete stabilization when compared with
those of 5 h incubation. These results suggest different binding kinetics, implying that this method may also provide kinetic information on active site binding of triclosan and NAD(P)^+.

In this study, we used Sypro Orange thermal shift on purified protein. With the recent development of cellular thermal shift assay\textsuperscript{27,28} it is certainly possible, using the principles discussed in this work, to apply cellular thermal shift assay on cells to screen for triclosan-resistant ENR/FabI mutants of species requiring enoyl-ACP linked substrate. This method may also be used to select effective triclosan analogues that, together with NAD(P)^+, bind to the active site of ENR/FabI to inhibit the enzymatic activity, and thus inhibit cell growth.

In summary, we have shown that the Sypro Orange-based thermal shift binding assay, with fluorescence-sensitivity, can be used easily and reliably to show the binding of the antibiotic triclosan in the presence of NAD(P)^+ to the active site of ENR/FabI enzymes, but not to a triclosan-resistant mutant. Thus, the method can be applied to FabI systems that require ACP-linked substrates without the need for the substrate to assay for activities. We have shown the lack of a thermal shift in a triclosan-resistant mutant. This approach offers a quick and convenient method to screen for triclosan-resistant mutations in ENR/FabI. This approach may also be applied to other antibiotics. In addition, it may also provide mechanistic insights into inhibition by studying the kinetics of the binding of triclosan to ENR/FabI.

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References


2. Wang, Y.; Shutao, M. Recent advances in inhibitors of bacterial fatty acid synthesis type II (FASII) system enzymes as potential antibacterial agents. ChemMedChem. 2013. 8, 1589-1608.


Figure Legends

Figure 1. The decrease of 250 uM NADH absorbance upon the addition of 200 uM crotonyl-CoA by 450 nM FtFabI WT with no triclosan (cross) and with 1.125 uM triclosan (asterisk). Also shown were those of 450 nM of FtFabI A92V with no triclosan (empty circle) and with 1.125 uM triclosan (filled circle). An increased concentration of FtFabI A92V (1 uM)
with no triclosan (empty square) and with 5 uM triclosan (filled square). The slope of the linear fit of the data points (ΔA_{340}/Δmin) was converted to a rate of NADH consumed, using the NADH extinction coefficient of 6220 M⁻¹ cm⁻¹. Specific activities for FtFabI (WT and A92V) were calculated as the rate of NADH consumed (umol/min) per milli-gram of FtFabI. For FtFabI WT, the activity was 0.66 umol min⁻¹ mg⁻¹. See text for details.

Figure 2. Representative thermal unfolding profiles of SaFabI WT and M99T samples, derived by monitoring the fluorescence emission intensity of Sypro Orange at 570 nm, with excitation at 472 nm. SaFabI WT (cross) (left panel), with a T_{mSO} of 40.5 °C and SaFabI WT incubated with 100 uM triclosan and 200 uM NADP⁺ for 5 h (diamond), with a T_{mSO} of 58.6 °C. We found that incubating triclosan and NADP⁺ for 30 min (triangle) was not sufficient for SaFabI, and the sample consisted of two populations of SaFabI, one without and one with triclosan/NADP⁺. The first transition temperature 40.9 °C was that of SaFabI without triclosan and NADP⁺, and the second transition temperature 60.9 °C was that of SaFabI with triclosan and NADP⁺. With 5 hr incubation, the transition temperature shifted to 58.6 °C in this typical paired run. SaFabI M99T (cross) (right panel), with T_{mSO} of 39.7 °C and SaFabI M99T with 100 uM triclosan and 200 uM NADP⁺ after 5 hr incubation (diamond), with T_{mSO} of 60.6 °C. The data from the sample with 30 min incubation (triangle) showed two transitions at 39.8 and 61.4 °C.

Figure 3. Representative thermal unfolding profiles of SaFabI A95V, I193S, and F204S samples, derived by monitoring the fluorescence emission intensity of Sypro Orange at 570 nm, with excitation at 472 nm. SaFabI A95V (cross) (left panel), with a T_{mSO} of 39.3 °C and SaFabI A95V incubated for 5 h with 100 uM triclosan and 200 uM NADP⁺ (open diamond) with a T_{mSO} of 40.1 °C. SaFabI I193S (cross) (middle panel), with a T_{mSO} of 40.3 °C and SaFabI I193S incubated for 5 h with 100 uM triclosan and 200 uM NADP⁺ (open diamond) with a T_{mSO} of 40.3 °C. SaFabI F204S (cross) (right panel), with a T_{mSO} of 41.2 °C and SaFabI F204S incubated for 5 h with 100 uM triclosan and 200 uM NADP⁺ (open diamond) with a T_{mSO} of 41.0 °C. No thermal shift was observed for SaFabI A95V, I193S, or F204S mutants.
Representative thermal unfolding profiles of *FtFabI* WT and A92V samples, derived by monitoring the fluorescence emission intensity of Sypro Orange at 570 nm, with excitation at 472 nm. The $T_{m}^{SO}$ of *FtFabI* WT (cross) (left panel) was 53.2 °C, and of *FtFabI* WT with 100 uM triclosan and 200 uM NAD$^+$ after 30 min incubation (triangle) was 60.3 °C, and after 5 hr (diamond) was 62.6 °C. For A92V *FtFabI* (cross) (right panel), the $T_{m}^{SO}$ was 51.8 °C, and for A92V *FtFabI* with 100 uM triclosan and 200 uM NAD$^+$ for 30 min (triangle), the $T_{m}^{SO}$ was 51.0 °C, and for 5 hr (diamond), the $T_{m}^{SO}$ was 52.1 °C.

Representative thermal unfolding profiles and $T_m$ values derived by monitoring CD signal at 222 nm from 25 - 75 °C of 15 uM *FtFabI* WT with no DMSO (empty circle) -- $T_m = 57.7 \pm 0.1$ °C, 0.1% DMSO (half-filled circle) -- $T_m = 57.7 \pm 0.1$ °C, 0.1% DMSO and Sypro Orange (filled circle) -- $T_m = 56.4 \pm 0.2$ °C and of 15 uM *SaFabI* WT with no DMSO (empty square) -- $T_m = 43.7 \pm 0.0$ °C, 0.1% DMSO (half-filled square) -- $T_m = 43.6 \pm 0.0$ °C and 0.1% DMSO and Sypro Orange (filled square) -- $T_m = 42.7 \pm 0.0$ °C. These results show little DMSO (0.1%) or Sypro Orange effect on the thermal unfolding of either *FtFabI* or *SaFabI*. 
Fig. 2

The figure shows temperature (Temp, °C) vs. fraction unfolded for two strains of SaFabi: WT and M99T. Two sets of data are plotted, each represented by different symbols and lines. The x-axis represents temperature ranging from 20 to 80 °C, while the y-axis represents the fraction unfolded ranging from 0.0 to 1.0. The left side of the figure presents the data for SaFabi WT, and the right side for SaFabi M99T.
Fig. 4

The figure shows the fraction of unfolded proteins (FiFabl WT and FiFabl A92V) plotted against temperature (Temp (°C)). The x-axis represents temperature ranging from 20 to 80°C, while the y-axis represents the fraction unfolded ranging from 0.0 to 1.0. The graph compares the unfolding behavior of the two protein variants across the temperature range.