JPP JOURNAL OF PROTEINS AND PROTEOMICS
4(2), December 2013, pp. 123-128

Research Article

BIOPHYSICAL CHARACTERIZATION OF A THERMOALKALOPHILIC ESTERASE FROM *Geobacillus* sp.

Liam M. Longo[1], Gulşah Şanlı-Mohamed[2] and Michael Blaber*[1]

[1]*Department of Biomedical Sciences, Florida State University, Tallahassee FL 32306-4300, USA and*
[2]*Department of Chemistry, Izmir Institute of Technology, Izmir, Turkey*

Abstract: Esterases are a class of enzyme with broad industrial applications in stereo-specific synthetic approaches to metabolic processing of drugs and antimicrobial agents. As such, preparation of an efficient esterase that is robust to denaturation under a wide range of temperatures and solvent conditions would be of great practical utility. To this end, an esterase cloned from the thermophilic bacteria *Geobacillus* sp. was subjected to biophysical characterization. In the presence of reducing agents (e.g., dithiothreitol), equilibrium studies of heat- and chemical-induced denaturation were apparently two-state and reversible. Derived parameters from unfolding studies using fluorescence and circular dichroism were similar, indicating that unfolding is largely cooperative. As predicted, the esterase is highly stable, with $T_{m\ app}$ = 75.8 °C and $\Delta G_{unf\ app}$ = –69.8 kJ/mol at 25°C. Refolding studies carried out at 25°C reveal the presence of one or more folding intermediates. Taken together, these results suggest that this esterase is an excellent candidate for use in industrial applications, and indicate that the systematic removal of Cys to recover reversible folding in the absence of reducing agents will be a feasible approach to further improving the utility of this enzyme.

Keywords: Protein stability; protein design; extremophile; thermophile

Introduction

Esterases (EC 3.1.1.1) are members of the hydrolase class of enzymes and catalyze the hydrolytic cleavage (or synthesis) of carboxylic ester bonds. Nearly ubiquitous in animals, plants, and microorganisms (Bornscheuer, 2002) esterases are known to possess a wide range of natural and non-natural substrates and consequently are of special interest to a variety of biotechnological applications (Bornscheuer *et al.*, 2002). Additionally, many esterases do not require cofactors for enzymatic efficiency or specificity and exhibit high regio- and stereoselectivity, making them ideal for stereospecific synthesis applications. Indeed, every year novel biotechnological applications are established using esterases, which often catalyze reactions of medical interest, such as the metabolic processing of drugs and antimicrobial agents (Margolin, 1993; Quax and Broekhuizen, 1994).

General catalytic rates are enhanced at higher temperatures, and enzymes from extremophiles often perform better in a number of commercial applications when compared to related mesophilic enzymes. Additionally, in some cases, thermophilic enzymes have been known to retain stability in organic solvents, making them of particular use to large-scale organic syntheses, or reactions where reduced water activity is desired. With such considerations in mind we report the initial biophysical characterization of an esterase ("gEst") cloned from *Geobacillus* sp., a thermophilic bacteria found at the Balçova geothermal site in Turkey. Although previous

Corresponding Author: **Michael Blaber**
E-mail: michael.blaber@med.fsu.edu

Received: November 10, 2013
Accepted: December 7, 2013
Published: December 14, 2013

reports have demonstrated the potential utility of this esterase in industrial synthesis, rigorous biophysical characterization has not yet been reported.

Materials and Methods

Protein Expression and Purification - *E.coli* BL21(DE3) harboring the IPTG inducible plasmid pET-28a(+) encoding for gEst was expressed and purified by $(His)_6$ nickel affinity chromatography (Tekedar and Sanli-Mohamed, 2011). Protein homogeneity was evaluated using Coomassie Blue visualized SDS-PAGE (Laemmli, 1970). Protein concentration was calculated with an extinction coefficient of 16,666 M^{-1} cm^{-1}, determined using the method of Gill and von Hipple (Gill and von Hippel, 1989). Purified gEst was dialyzed into 20 mM N-(2-acetamido) iminodiacetic acid (ADA), 100 mM NaCl, 2 mM DTT, pH 6.5 ("ADA Buffer") for biophysical characterization. ADA is compatible with a wide range of biophysical techniques (ADA does not have significant UV absorbance; the pK_a of ADA is largely temperature independent) and is thus ideal for characterization of protein stability. Conditions of low pH were used to minimize deprotonation of Cys residues, which is a key step in the formation of mixed thiols (Jocelyn, 1972).

Heat Denaturation - 10 μM samples of gEst in ADA Buffer were equilibrated for 10 minutes at 20°C. After equilibration, samples were heated at 0.25°C / min. Unfolding was monitored by fluorescence (λ_{ex} = 295 nm, λ_{em} = 350 nm; 5 nm slit widths) using a Cary Eclipse spectrophotomer equipped with a Peltier temperature control. The resulting thermal unfolding curves were fit to a six-parameter, two-state unfolding model (which calculates the ΔG(T) function assuming ΔCp = 0) (Shih *et al.*, 1995). Curve fitting was performed by the nonlinear, least squares fitting program, DataFit (Oakdale Engineering). Reported parameters are the average and standard deviation of three independent experiments.

Chemical Denaturation - Isothermal equilibrium denaturation experiments were performed at 25°C using guanidinium hydrochloride (GuHCl) as the denaturant. The concentration of a stock solution of GuHCl in ADA Buffer was determined using refractometry (Nozaki, 1972). 10 μM samples of gEst in ADA Buffer were equilibrated overnight at room temperature in the presence of increasing concentrations of GuHCl. Unfolding of individual samples was monitored by both fluorescence and circular dichroism (CD). As above, fluorescence measurements were collected using a Cary Eclipse spectrophotometer equipped with a Peltier temperature control unit (λ_{ex} = 295 nm, λ_{em} = 304 - 500 nm; 5 nm slit widths). The resulting fluorescence spectra were buffer-subtracted and integrated to generate an unfolding curve. CD measurements were collected on a Jasco J-815 CD spectropolarimeter equipped with a Peltier temperature control unit. CD was measured between 215 nm and 235 nm, with the signal at 222 nm used to generate the unfolding curve. Throughout the course of the CD experiment detector gain did not exceed 800 V. Chemical denaturation profiles were analyzed by fitting to a six-parameter, two-state model (Santoro and Bolen, 1988) using the nonlinear, least squares fitting program, DataFit (Oakdale Engineering). Reported parameters are the average and standard deviation of three independent experiments.

Folding and Unfolding Kinetics - Refolding and unfolding kinetic data of gEst were collected at 25°C and monitored by fluorescence. Unfolding kinetics, which are monophasic, were collected by manual mixing using a Cary Eclipse spectrophotometer equipped with a Peltier temperature control unit (λ_{ex} = 295 nm, λ_{em} = 350 nm; 5 nm slit widths). In each case, gEst samples in ADA Buffer were rapidly diluted into denaturing conditions (3.0-5.5 M GuHCl in ADA Buffer) resulting in a final concentration of 10 μM gEst. To ensure characterization over essentially the entire unfolding process, unfolding reactions were monitored for at least 4 half-lives (~94% of the total unfolding process). The resulting curves were fitted to a simple exponential decay function to determine the unfolding rate constant.

Refolding kinetics, which are biphasic, required stopped-flow methods for accurate determination of both folding rate constants. These data were collected on an SX-20 stopped-flow system (Applied Photophysics), λ_{ex} = 295 nm, 305 nm emission filter, and with an attached

temperature-controlled water bath. Samples were equilibrated overnight in 3.5 M GuHCl in ADA Buffer before rapidly diluting into refolding conditions (0.32-2.40 M GuHCl). Refolding reactions were monitored for at least 4 half-lives and the resulting curves were fitted to a biexponential decay function to determinethe folding rate constants.

Results and Discussion

Fluorescence and Circular Dichroism Spectra

Both the fluorescence and the CD profiles of gEst change dramatically upon addition of denaturant (Figure 1). Native state gEst (0M GuHCl) exhibits a strong fluorescence signal with a maximum at 340 nm; the fluorescence signal in 6M GuHCl is significantly attenuated and red-shifted, with a maximum around 359 nm. The observed fluorescence profiles are consistent with the exposure of buried Trp residues to polar solvent upon unfolding (Vivian and Callis, 2001). A crystal structure of the closely related esterase *G. stearothermophilus* with 97% amino acid identity predicts that gEst is composed of six α-helices; indeed, the CD spectrum of native gEst (0M GuHCl; Figure 1b) displays the characteristic peak at 222 nm, indicating the presence of α-helices, which is lost in 6M GuHCl denaturant—consistent with unfolding. Critically, reversible refolding of gEst was observed only in the presence of 2 mM DTT; buffer formulated with 2 mM DTT results in ~90% recovery of fluorescence signal upon refolding compared to <20% signal recovery in the absence of DTT (overnight incubation; data not shown). As such, all characterization of gEst was performed in the presence of 2 mM DTT and under reversible conditions. The above result strongly suggests that gEst contains reactive thiols that contribute to irreversible denaturation; a Cys-free form of gEst would therefore be a superior candidate to the wild-type for many industrial applications (Lee and Blaber, 2009).

Heat Denaturation

Heat denaturation of gEst followed by fluorescence is described well by a simple two-state unfolding model (Figure 2b). The apparent midpoint of the unfolding transition ($T_{m\ app}$) occurs at 75.8 ± 0.3 °C, about 15 °C above the temperature of the Balçova geothermal site (Turkey) from which *Geobacillus* sp. was isolated. The enthalpy of denaturation at the T_m was determined to be 763 ± 35 kJ/mol. At temperatures above 88 °C, gEst undergoes irreversible aggregation and precipitates. These results are consistent with previous reports that indicate the activity of gEst sharply declines after a 1 hr incubation at temperatures higher than 60 °C (Gulay and Sanli-Mohamed, 2012). Notably, the aforementioned heat incubation studies were performed in the absence of DTT, for which folding of gEst is non-reversible (Gulay and Sanli-Mohamed, 2012).

Chemical Denaturation

Chemical denaturation by guanidinium hydrochloride (GuHCl) was performed at 25 °C

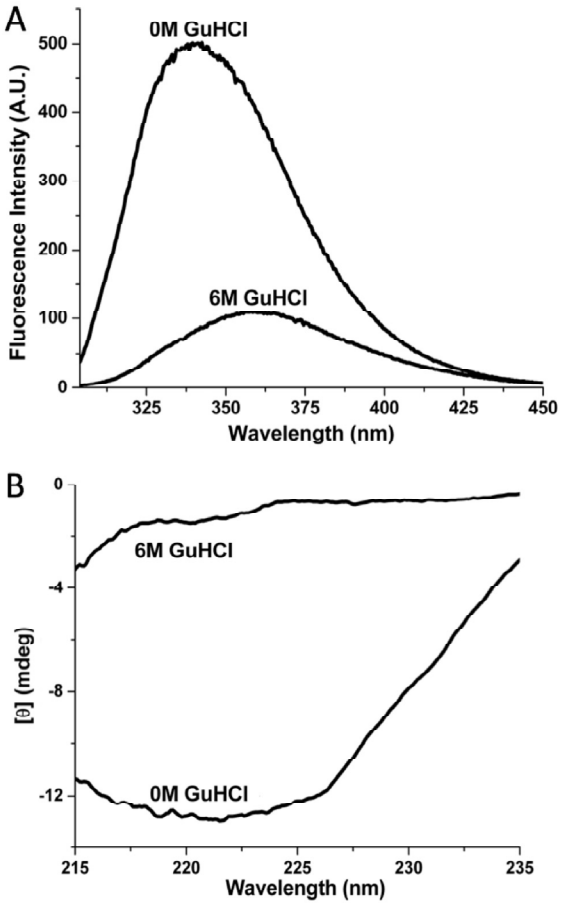
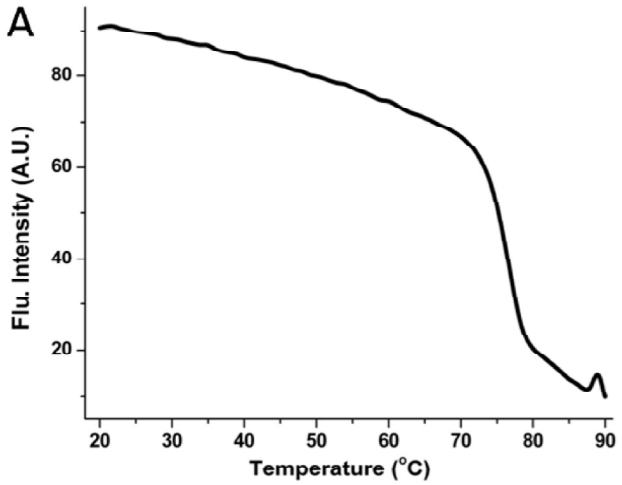


Figure 1: **Representative unfolding profiles of gEst monitored by fluorescence (Panel A) and circular dichroism (Panel B). Denaturant-induced unfolding of gEst is associated with significant spectral changes (see text).**



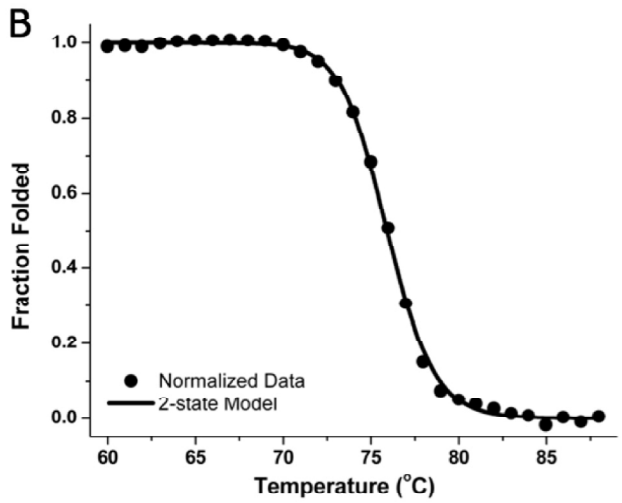
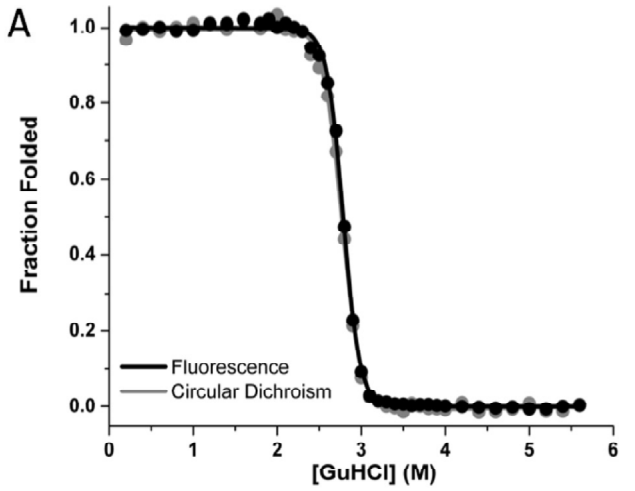


Figure 2: **Thermal unfolding of gEst monitored by fluorescence (Panel A) and subsequent modeling (Panel B). Samples were heated at a rate of 0.25 °C/min to ensure that the protein was fully equilibrated at all temperatures. Data represents the average of three separate experiments; fraction folded was calculated using the equation $f_N = (S_{obs} - S_D) / (S_N - S_D)$, where f_N is the fraction native, S_{obs} is the observed signal, S_D is the signal of the denatured state, and S_N is the signal of the native state. Native state and denatured state signals were taken to be linear functions of temperature.**



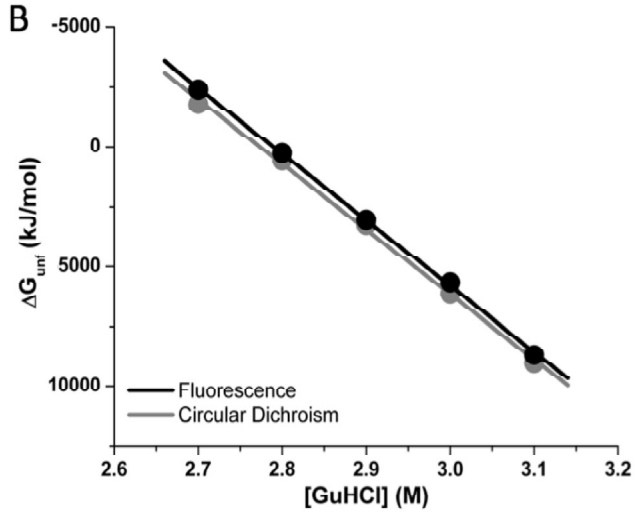


Figure 3: **Isothermal equilibrium denaturation of gEst at 25 °C. Denaturation curves generated from fluorescence and CD (Panel A) overlay well, indicating that the unfolding of gEst is concerted. Plots of ΔG as a function of denaturant about the apparent midpoint of denaturation highlight the similarity of the two spectral probes (ΔG values calculated from raw data not model fits). Each curve represents the average of three independent experiments; fraction folded was calculated using the equation $f_N = (S_{obs} - S_D) / (S_N - S_D)$, where f_N is the fraction native, S_{obs} is the observed signal, S_D is the signal of the denatured state, and S_N is the signal of the native state. Native state and denatured state signals were taken to be linear functions of denaturant concentration.**

and monitored by both fluorescence and CD (Figure 3a, Table 1). In both cases, the resulting unfolding profiles exhibited excellent agreement with a simple two-state model of unfolding. Fitted parameters from the fluorescence (a measure of tertiary structure formation) and CD (a measure of secondary structure formation) unfolding curves were similar, suggesting that gEst unfolding is highly cooperative (i.e., global). As predicted, gEst is highly stable, with a $\Delta G_{unf\ app}$ = 69.8 kJ/mol (Flu) or 63.0 kJ/mol (CD) in 0M

Table 1
Fitted parameters from GuHCl induced denaturation of gEst at 25 °C

Spectroscopic Probe	$\Delta G_{unf\ app\ H2O}$ *(kJ/mol)*	m_{app} *(kJ/mol/M)*	c_m *(M)*
Fluorescence	-69.8 ± 3.7	25.1 ± 1.2	2.79 ± 0.02
Circular Dichroism	-63.0 ± 5.7	22.7 ± 1.8	2.77 ± 0.03

GuHCl. Differences in the ΔG at 0M denaturant between CD and fluorescence result primarily from the long extrapolation to 0M GuHCl from the midpoint of denaturation [$c_{m\,app}$ = 2.79 M (Flu) or 2.77 M (CD)]; a plot of ΔG as a function of [GuHCl] in the region surrounding the midpoint of denaturation (i.e., where the data is most accurate; Figure 3b) better highlights the similarity between the two probes. In this region, the difference between calculated values of ΔG is less than 0.4 kJ/mol (that is, within experimental error in this region).

Folding and Unfolding Kinetics

Unfolding of gEst by GuHCl at 25 °C was monophasic at all concentrations of GuHCl; refolding, however, was biphasic until 2.1 M GuHCl (Figure 4). Additionally, both the folding and unfolding arms of the chevron plot exhibit significant deviation from two-state behavior (i.e., both arms are non-linear). At low concentrations of GuHCl, the slope of the fast phase of the refolding arm is positive (indicating that denaturant *increases* the rate of folding) whereas the slope of the slow phase of the refolding arm is relatively flat (that is, denaturant *independent*) until about 1.5 M GuHCl. These features are consistent with multi-state folding; specifically, positive slopes on the refolding arm of the chevron plot often occur when a compact intermediate state in fast equilibrium with the denatured state is populated (Otzen and Oliveberg, 1999). The unfolding arm of the chevron plot has noticeable downward curvature. The underpinning physical interpretation of curved chevron plot arms, however, is ambiguous in the absence of additional detailed folding data (Sanchez and Kiefhaber, 2003); however, the steep slope of both the folding and unfolding arms indicate a high-degree of folding cooperativity. Kinetic experiments were performed at 25°C, well below the natural environment within which gEst would operate; thus, the observed folding complexity may change as the temperature is raised—in particular, it is possible that a folding intermediate responsible for biphasic folding at 25°C may be destabilized at higher temperatures, yielding monophasic folding (Blaber *et al.*, 1999).

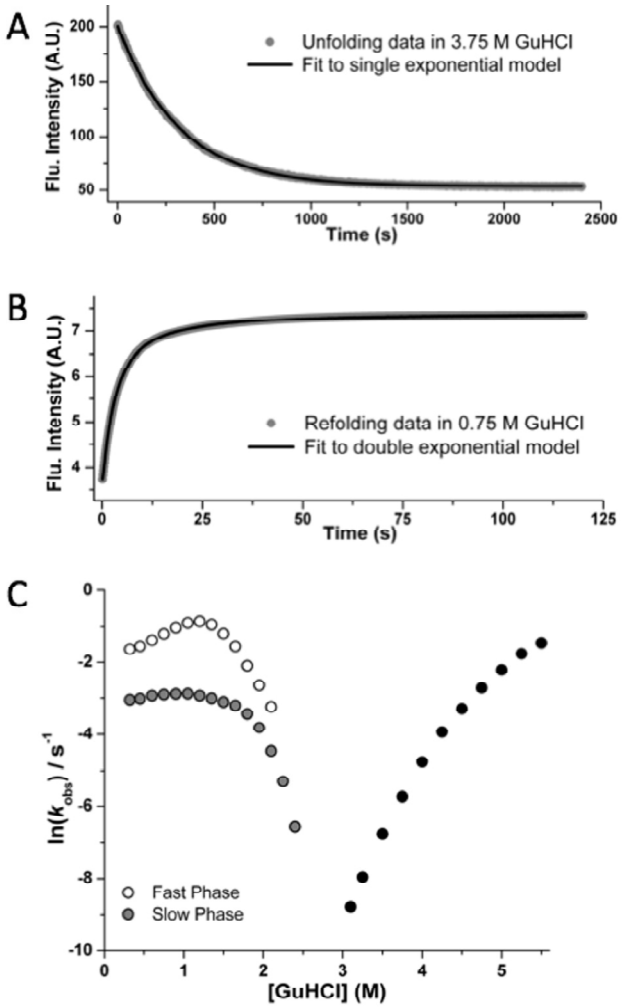


Figure 4: **Representative unfolding and refolding kinetic traces with fitted models (Panels A and B, respectively). Chevron plot of gEst folding and unfolding kinetics at 25 °C (Panel C). Folding and unfolding kinetics of gEst (247 amino acids) is markedly non-two-state (see text), as is common for proteins over ~150 amino acids (Jackson, 1998).**

Conclusions

Biophysical characterization of the equilibrium and kinetic properties of gEst indicate it is a thermophile with robust $\Delta G_{unf\,app}$ of ~65 kJ/mol; thus, there is considerable thermodynamic stability—useful for either high-temperature esterase catalysis or, conversely, the ability to accommodate secondary mutations for purposes of protein engineering to enhance functionality. As regards this latter point, the functional half-life of gEst is potentially severely limited by thiol-mediated irreversible denaturation. gEst will serve as a critical point of departure for the design of 2nd generation esterases with properties suitable

for industrial applications—initially targeting Cys-free variants.

Acknowledgements

LML gratefully acknowledges FSU COM for financial support during this project.

References

Blaber, S.I., Culajay, J.F., Khurana, A. and Blaber, M. (1999). Reversible thermal denaturation of human FGF-1 induced by low concentrations of guanidine hydrochloride. Biophys. J. *77*, 470-477.

Bornscheuer, U.T. (2002). Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS Microbiol. Rev. *26*, 73-81.

Bornscheuer, U.T., Bessler, C., Srinivas, R. and Krishna, S.H. (2002). Optimizing lipases and related enzymes for efficient application. Trends Biotech *20*, 433-437.

Gill, S.C. and von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. *182*, 319-326.

Gulay, S. and Sanli-Mohamed, G. (2012). Immobilization of thermoalkalophilic recombinant esterase enzyme by entrapment in silicate coated Ca-alginate beads and its hydrolytic properties. Int J Biol Macromol *50*, 545-551.

Jackson, S.E. (1998). How do small single-domain proteins fold? Folding Design *3*, R81-R91.

Jocelyn, P.C. (1972). Biochemistry of the SH group; the occurrence, chemical properties, metabolism and biological function of thiols and disulphides, Academic Press, New York..

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lee, J. and Blaber, M. (2009). The interaction between thermostability and buried free cysteines in regulating the functional half-life of fibroblast growth factor-1. J. Mol. Biol. *393*, 113-127.

Margolin, A.L. (1993). Enzymes in the synthesis of chiral drugs. Enzyme Microbial Tech. *15*, 266-280.

Nozaki, Y. (1972). The preparation of guanidine hydrochloride. Methods Enzymol *26*, 43-50.

Otzen, D.E. and Oliveberg, M. (1999). Salt-induced detour through compact regions of the protein folding landscape. Proc. Natl. Acad. Sci. USA *96*, 11746-11751.

Quax, W.J. and Broekhuizen, C.P. (1994). Development of a new Bacillus carboxyl esterase for use in the resolution of chiral drugs. App. Microbiol. Biotech. *41*, 425-431.

Sanchez, I.E. and Kiefhaber, T. (2003). Hammond behavior versus ground state effects in protein folding: Evidence for narrow free energy barriers and residual structure in unfolded states. J. Mol. Biol. *327*, 867-884.

Santoro, M.M., and Bolen, D.W. (1988). Unfolding Free-Energy Changes Determined by the Linear Extrapolation Method .1. Unfolding of Phenylmethanesulfonyl Alpha-Chymotrypsin Using Different Denaturants. Biochemistry *27*, 8063-8068.

Shih, P., Holland, D.R. and Kirsch, J.F. (1995). Thermal stability determinants of chicken egg-white lysozyme core mutants: hydrophobicity, packing volume, and conserved buried water molecules. Protein Sci. *4*, 2050-2062.

Tekedar, H.C. and Sanli-Mohamed, G. (2011). Molecular cloning, over expression and characterization of thermoalkalophilic esterases isolated from Geobacillus sp. Extremophiles *15*, 203-211.

Vivian, J.T. and Callis, P.R. (2001). Mechanisms of tryptophan fluorescence shifts in proteins. Biophys. J. *80*, 2093-2109.