Incorporation of Trifluoromethionine into a Phage Lysozyme: Implications and a New Marker for Use in Protein ^{19}F NMR†

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ABSTRACT: Much interest is currently focused on understanding the detailed contribution that particular amino acid residues make in protein structure and function. Although the use of site-directed mutagenesis has greatly contributed to this goal, the approach is limited to the standard repertoire of twenty amino acids. Fluorinated amino acids have been utilized successfully to probe protein structure and dynamics as well as point to the importance of specific residues to biological function. In our continuing investigations on the importance of the amino acid methionine in biological systems, the successful incorporation of L-*S*-(trifluoromethyl)homocysteine (L-trifluoromethionine; L-TFM) into bacteriophage λ lysozyme (LaL), an enzyme containing three methionine residues, is reported. The L isomer of TFM was synthesized in an overall yield of 33% from *N*-acetyl-D,L-homocysteine thiolactone and trifluoromethyl iodide. An expression plasmid giving strong overproduction of LaL was prepared and transformed into an *Escherichia coli* strain auxotrophic for methionine permitting the expression of LaL in the presence of L-TFM. The analogue would not support growth of the auxotroph and was found to be inhibitory to cell growth. However, cells that were initially grown in a Met-rich media followed by protein induction under careful control of the respective concentrations of L-Met and L-TFM in the media, were able to overexpress TFM-labeled LaL (TFM-LaL) at both high (70%) and low (31%) levels of TFM incorporation. TFM-LaL at both levels of incorporation exhibited analogous activity to the wild type enzyme and were inhibited by chitooligosaccharides indicating that incorporation of the analogue did not hinder enzyme function. Interestingly, the ^{19}F solution NMR spectra of the TFM-labeled enzymes consisted of four sharp resonances spanning a chemical shift range of 0.9 ppm, with three of the resonances showing very modest shielding changes on binding of chitopentaose. The ^{19}F NMR analysis of TFM-LaL at both high and low levels of incorporation suggested that one of the methionine positions gives rise to two separate resonances. The intensities of these two resonances were influenced by the extent of incorporation which was interpreted as an indication that subtle conformational changes in protein structure are induced by incorporated TFM. The similarities and differences between Met and TFM were analyzed using *ab initio* molecular orbital calculations. The methodology presented offers promise as a new approach to the study of protein−ligand interactions as well as for future investigations into the functional importance of methionine in proteins.

Over the past several years our appreciation of the important, although sometimes subtle, roles that the amino acid methionine plays in protein structure and function has dramatically increased (Gellman, 1991; Viguera & Serrano, 1995). Although methionine is the third least-abundant amino acid found in proteins (Doolittle, 1989), its presence serves a variety of roles in biological systems. Methionine has been directly implicated in a variety of substrate recognition processes. A number of proteins appear to utilize methionine's flexibility around its χ_3 torsion angle (Gellman, 1991), which makes it more flexible than other hydrophobic amino acids such as leucine and isoleucine (Janin et al., 1978), as well as its polarizable sulfur atom for use in recognition and binding events. Methionine residues are now known to act as important structural elements in the sequence-independent recognition of nonpolar peptide and protein surfaces (Gellman, 1991). Signal recognition particle 54 (SRP54) is responsible for the recognition and binding of a variety of signal sequences found in nascent secretory and membrane proteins. The recognition of these signal peptides by SRP54 is believed to be due, in part, to the presence in SRP54 of three methionine rich amphiphilic helices (M-regions) which form the basis of a hydrophobic binding pocket (Bernstein et al., 1989; Lutcke et al., 1992). In the case of calmodulin, which binds several different peptides unrelated in sequence, recent X-ray (Meador et al., 1992) and NMR (Ikura et al., 1992; Zhang & Vogel, 1994) studies of calmodulin−peptide complexes indicate that all nine methionine residues in the protein interact directly with the peptide ligand. A similar interaction is believed to occur in the case of a related protein, troponin C (Lin et al., 1994).

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[1] Abbreviations: ESMS, electrospray mass spectrometry; $(GlcNAc)_n$, $\beta(1\rightarrow4)$-linked *n*-mer of *N*-acetyl-D-glucosamine; IPTG, isopropyl β-D-thiogalactoside; LaL, λ lysozyme; L-Met, L-methionine; $M9_{Glu}$, $M9_{Glu,amp}$, M9 minimal medium supplemented with glucose (Glu) or glucose and ampicillin (Glu,amp); NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; SRP, signal recognition particle; TFM, *S*-(trifluoromethyl)-homocysteine, trifluoromethionine; TFM-LaL, TFM-labeled LaL; TLC, thin-layer chromatography; *wt*, wild type.

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In addition, a recent report has implicated methionine residues in the collagen/gelatin binding of plasma fibronectin (Miles & Smith, 1993).

As important for recognition and binding but not perhaps as dramatic are the proteins that appear to utilize single methionine residues in their active sites for substrate recognition. Methionine 108 in the arabinose-binding protein from *Escherichia coli* has been shown by X-ray crystallography and site-directed mutagenesis to play a direct role in binding of this monosaccharide (Vermersch et al., 1991). The preference of the enzyme for arabinose (arabinose > galactose ≫ fucose) is altered in the M108L mutant (galactose ≫ fucose > arabinose). It is believed that this selectivity is governed by both an "apolar−apolar" as well as a "polar−apolar" interaction with the sugar and the methionine residue. A methionine, Met 502, has also been found to be near the active site in the crystal structure of *E. coli* β-galactosidase (Jacobson et al., 1994). In addition, the thioether moiety of methionine functions as a ligand to iron centers such as in various *c* cytochromes (Senn et al., 1983; Wallace & Clark-Lewis, 1992; Ubbink et al., 1994) and to copper centers such as is found in plastocyanin (Collyer et al., 1990; Guckert et al., 1995), several azurins (Murphy et al., 1993; Salgado et al., 1996), nitrite reductase (Godden et al., 1991), ascorbate oxidase (Messerschmidt et al., 1992), dopamine β-hydroxylase (Reedy & Blackburn, 1994), and peptidylglycine α-hydroxylating monooxygenase (Eipper et al., 1995).

The ability of methionine residues to undergo facile oxidation to the sulfoxide level may play a role in the control of protein function. Several proteins are known to have altered biological activities when one or more methionine residues have been oxidized (Brot & Weissbach, 1991; Kachurin, 1995). These effects, such as in the case of the lung elastase α-1-proteinase inhibitor (α1-PI), can become clinically relevant (Travis & Salvesen, 1983). A protein-methionine-*S*-oxide reductase (EC 1.8.4.6) appears to mediate thioredoxin-dependent reduction of methionine sulfoxide residues in proteins (Moskovitz et al., 1995). Although the effects of methionine oxidation are well documented, the extent of the contribution of methionine oxidation−reduction in the control of enzyme activity *in vivo* is currently unknown (Vogt, 1995). A recent report on the possible control of calmodulin activity by *in vivo* oxidation of one of its methionine residues would appear to favor this novel mechanism in the modulation of protein activity (Yao et al., 1996).

With a natural abundance of 100%, a sensitivity to NMR detection of 83% that of ^{1}H and a high sensitivity of ^{19}F shifts to the surrounding environment, ^{19}F NMR is an extremely valuable technique for investigating protein structure and dynamic changes upon ligand binding (Sykes & Hull, 1978; Gerig, 1989, 1994; Luck & Falke, 1991a−c; Hoeltzli & Frieden, 1994; Lian et al., 1994; Danielson & Falke, 1996). The fluorine atom is relatively nonsterically demanding and in many cases can replace a hydrogen atom in biological systems with minimal structural change. The availability of amino acid auxotrophic strains of microorganisms (Sykes & Weiner, 1980) and modern recombinant expression systems have permitted the introduction of fluorinated amino acids into a wide variety of proteins (Gerig, 1994). Interestingly, in spite of these considerations, to our knowledge the ^{19}F protein NMR studies reported to date have centered predominantly upon the utilization of fluorinated analogues of the aromatic amino acids tryptophan (4-, 5-, and 6-fluoro), phenylalanine (2-, 3-, and 4-fluoro), and tyrosine (3-fluoro) (Gerig, 1994). A paucity of studies dealing with the application of other fluorinated amino acids to ^{19}F protein NMR currently exists. A report on the X-ray structure of an inactive semisynthetic ribonuclease-S containing a 4-F-histidine residue has been published although no ^{19}F NMR experiments were presented (Taylor et al., 1981). As well the incorporation of 5-F-leucine into dihydrofolate reductase has been reported (Feeney et al., 1996).

Recently we reported our synthetic efforts to prepare a variety of fluorinated methionine and methionine salvage pathway analogues (Houston & Honek, 1989; Houston et al., 1991). Because of our current interest in investigating the subtle functions methionine plays in protein structure and function, as well as to develop an additional approach to the study of ligand−protein interactions by ^{19}F NMR, we have investigated the incorporation of one such analogue, L-*S*-trifluoromethylhomocysteine (L-trifluoromethionine; L-TFM), into the lysozyme from bacteriophage lambda (LaL). Our investigation demonstrates the usefulness of this unique ^{19}F probe in studying protein−ligand interactions in general and suggests this approach may prove valuable for the specific study of the function of methionine residues in biological systems.

MATERIALS AND METHODS

Materials. Trifluoromethyl iodide and *N*-acetyl-D,L-homocysteine thiolactone were purchased from Aldrich (Milwaukee, WI). Acylase I (Grade I, from porcine kidney) and L-Met were from Sigma (Mississauga, ON), while chitooligosaccharides (fine grade) were obtained from Siekagaku America Inc. (Rockville, MD). Silica gel (70−230 Mesh) was obtained from EM Science (Gibbstown, NJ), while thin-layer chromatography (TLC) was performed on 0.25 mm Alugram Sil G/UV$_{254}$ plates from Macherey-Nagel (Dueren, Germany). The bacterial strains *E. coli* BL21(DE3) and methionine auxotroph B834(DE3) and plasmid pET-22b are products of Novagen (Madison, WI). Reagents for performing PCR and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Oligonucleotide primers were prepared by Dr. Reggie Lo (Department of Microbiology, University of Guelph) and DNA sequencing was a service provided by the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, ON).

Synthesis of L-*S-(Trifluoromethyl)homocysteine (*L-*TFM)*. The procedure is a slight modification of that previously developed (Houston & Honek, 1989; Houston, 1992).

(1) N-Acetyl-D,L-*S-(trifluoromethyl)homocysteine Methyl Ester*. Sodium metal (1.80 g, 78.3 mmol) was added over a 10 min period in small portions to anhydrous methanol (155 mL) cooled on ice under argon. When all the sodium had dissolved, *N*-acetyl-D,L-homocysteine thiolactone (5.0 g, 31.4 mmol) was added at once, the ice bath was removed, and the solution was stirred for 40 min. A dry ice/acetone condenser was attached to the flask, trifluoromethyl iodide (18.5 g, 94.4 mmol) was added to the solution over 15 min, and the solution was irradiated by three long-wave ultraviolet lamps. After 1 h, an additional amount of trifluoromethyl iodide (6.5 g, 33.2 mmol) was introduced into the flask over 5 min and the solution was irradiated for an additional 1 h.

The solvent was removed *in vacuo* and the oily residue was taken up in CH_2Cl_2 (300 mL) and washed successively with 5% $NaHCO_3$ (100 mL) and brine (125 mL). The organic layer was dried over $MgSO_4$ and filtered, and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel using ethyl acetate (EtOAc)/hexanes (3:1) yielding 6.46 g (24.9 mmol, 79% yield) of a clear light amber oil. R_f (EtOAc/hexanes 3:1; silica) 0.38; ^{1}H NMR (250 MHz, $CDCl_3$) δ 6.69 (d, 1H, J = 7.8 Hz, N*H*COCH$_3$), 4.64 (dt, 1H, J = 7.8 Hz, 5.0 Hz, CHα), 3.69 (s, 3H, CO_2CH_3), 2.85 (t, 2H, J = 7.5 Hz, $CH_2\gamma$), 2.28−1.90 (m, 2H, $CH_2\beta$), 1.97 (s, 3H, NHCOC*H*$_3$); ^{13}C NMR (62.2 MHz, $CDCl_3$) δ 171.9, 170.5 (NH*C*OCH$_3$, *C*O$_2$CH$_3$), 130.8 (q, J_{CF} = 306.3 Hz, CF_3), 52.5 (CHα), 51.0 (CO_2CH_3), 32.7 ($CH_2\beta$), 25.9 ($CH_2\gamma$), 22.8 (NHCO*C*H$_3$).

(2) L-*S-(Trifluoromethyl)homocysteine*. *N*-Acetyl-D,L-*S*-(trifluoromethyl)homocysteine methyl ester (6.46 g, 24.9 mmol) was dissolved in methanol (75 mL), and then Milli-Q water (75 mL) was added. The solution was cooled in an ice bath, and powdered NaOH (1.10 g, 27.5 mmol) was added. The solution was stirred on ice for 20 min and additionally at room temperature for 1 h. The solvent was removed *in vacuo*, and the resulting sodium salt of *N*-acetyl-D,L-*S*-(trifluoromethyl)homocysteine was dissolved in degassed Milli-Q water (150 mL), and the pH was brought to 7.4 with dilute HCl. Acylase I (10 mg, 19 900 U) was added and the solution was stirred gently under nitrogen at 25 °C for 8 h. Following 2 and 4 h, the solution was again degassed and the pH was adjusted to 7.4 with 0.1 N NaOH. The pH of the solution was brought to 5.0 with concentrated HCl. Decolorizing charcoal was added, and the solution was heated at 60 °C for 5 min and then filtered. The filtrate was adjusted to pH 1.4 with concentrated HCl and extracted with EtOAc (160 mL). The aqueous layer was applied to a Dowex 50W-X8 (H^+ form) column and eluted with Milli-Q water (pH 5−6) until the effluent was at pH 5−6. The column was then eluted with 1 M NH_4OH and fractions containing the product (monitored by TLC) were pooled and applied to a Lobar reverse phase C-18 column (Lichroprep RP-18, Merck). Elution was achieved sequentially with Milli-Q water, 10% and then 20% CH_3CN and fractions containing the product were pooled and lyophilized giving 2.11 g (10.4 mmol, 42% yield) of a white powder. Mp 227−229 °C [literature mp 229−230 °C decomp (Houston & Honek, 1989); 230 °C (Dannley & Taborsky, 1957)]; R_f (butanol−H_2O−acetic acid 4:1:1, silica) 0.46; ^{1}H NMR (250 MHz, D_2O) δ 3.54 (t, 1H, J = 6.5 Hz, CHα), 2.90 (t, 2H, J = 7.7 Hz, $CH_2\gamma$), 2.14−1.91 (m, 2H, $CH_2\beta$); ^{13}C NMR (62.2 MHz, D_2O): δ 172.2 (CO_2H), 131.1 (q, J_{CF} = 306.0 Hz, CF_3), 52.3 (CHα), 30.8 ($CH_2\beta$), 25.5 ($CH_2\gamma$); ^{19}F NMR (376 MHz, 50 mM potassium phosphate pH 7.0) δ −40.05. ESMS: exact mass calcd. for $C_5H_8F_3SNO_2$, 203.0228; found, 203.1.

Construction of Plasmid pLR102. A suitable plasmid for the overproduction of LaL was prepared by PCR. The template used was the LaL expression plasmid pHDM10 (Duewel et al., 1995). Primers were designed to introduce a 5′ *Kpn*I restriction site and a *Nde*I restriction site spanning the ATG start codon and *Bam*HI and *Hin*dIII restriction sites immediately 3′ to the termination codon. The synthetic primers used were primer 1, 5′-CCA**GGTACC***CATATG*GTAGAAATCAATAATCAA-3′, and primer 2, 5′-CCA**AAGCTT***GGATCC*TCATACATCAATCTCTCTGAC-3′. The *Kpn*I and *Nde*I sites (primer 1) and the *Hin*dIII and *Bam*HI sites (primer 2) are boldface and italicized, respectively, and sequences complimentary to the LaL sense (primer 1) and antisense (primer 2) strands are underlined. PCR was performed using a MiniCycler thermal unit (MJ Research, Inc.). The reaction mixture contained 1 unit of $Vent_R$ DNA polymerase, 4.5 ng of template DNA, approximately 40 μM of each primer and 200 μM of each dNTP in 80 μL of Vent Buffer. The sample was allowed to undergo 30 cycles of denaturation (90 s at 94 °C), annealing (60 s at 55 °C), and extension (30 s at 72 °C). The amplified product fragment was treated with *Kpn*I and *Hin*dIII and ligated into pUC18 cut with the same restriction enzymes. The ligation was transformed into $CaCl_2$-treated *E. coli* DH5α (Sambrook et al., 1989). Selected transformants were screened by restriction analysis to confirm the presence of the PCR product insert band. Plasmid DNA was isolated from several transformants; inserts were excised with *Nde*I and *Bam*HI, isolated, and ligated into pET-22b restricted with the same enzymes. The resulting plasmids were transformed into *E. coli* BL21(DE3) and, after IPTG induction, assayed for lysozyme activity (Duewel et al., 1995). One plasmid that had high activity was named pLR102. The lysozyme gene was sequenced in both directions from pLR102 to ensure that no mutations had occurred during the PCR reaction. The plasmid pLR102 was transformed into the methionine auxotroph *E. coli* B834(DE3) for L-TFM incorporation.

Expression and Purification of TFM-LaL. Unless otherwise stated, cells were grown in M9 minimal medium supplemented with 0.4% glucose and 40 mg/L ampicillin ($M9_{Glu,amp}$). *E. coli* B834(DE3)/pLR102 was grown at 37 °C in $M9_{Glu,amp}$ supplemented with 0.1 mM L-Met to an absorbance of 0.65 at 600 nm. Cells were centrifuged (9950*g*), washed with $M9_{Glu,amp}$ and then resuspended in one-half the original culture volume of $M9_{Glu,amp}$ containing 0.75 mM IPTG and either 1.0 mM L-TFM for high levels of incorporation or 1.0 mM L-TFM and 20 μM L-Met for low levels of incorporation and grown at 37 °C for 9.5 h. Cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate (pH 7) and disrupted either by sonication or passage through a French press (10 000 psi). Cell debris was removed by centrifugation (37000*g*). Lysozyme was purified by sequential chromatography over S-Sepharose Fast Flow, Mono-S, and Phenyl-Superose (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden), then dialyzed against 5 mM phosphate buffer (pH 7), and lyophilized as described previously (Duewel et al., 1995).

Optimization of Conditions Yielding Maximal TFM-LaL Expression and Desired Incorporation Levels. A culture of B834(DE3)/pLR102 was grown in $M9_{Glu,amp}$ supplemented with 0.1 mM L-Met, washed and resuspended in $M9_{Glu,amp}$ as described above and aliquoted into separate samples.

For High Levels of Incorporation. Sample aliquots were supplemented with L-TFM (0, 0.01, 0.1, 1.0, 2.0, or 5.0 mM) and 0.75 mM IPTG and incubated at 37 °C. At post-induction times of 4, 9.5, and 18 h, 0.5 mL aliquots of each were removed, centrifuged, and taken up in 50 μL protein loading buffer. Samples were analyzed by SDS−PAGE and the intensity of the LaL band (at approximately 18 kDa) was visually compared to determine which conditions afforded maximal expression.

Table 1: Effect of Various Concentrations of L-Met or L-TFM Alone or in Concert on the Growth of *E. coli* B834 (DE3) Harboring pLR102

	Absorbance (600 nm)[b]													
L-Met (mM)	0	0.01	0.05	0.5	0	0	0	0	0.01	0.05	0.5	0.01	0.05	0.5
L-TFM (mM)	0	0	0	0	0.01	0.1	1.0	5.0	1.0	1.0	1.0	5.0	5.0	5.0
growth time (h)[a]														
3	0.041	0.208	0.365	0.365	0.046	0.046	0.057	0.049	0.217	0.360	0.362	0.052	0.067	0.135
6	0.047	0.225	0.869	1.234	0.051	0.051	0.067	0.038	0.228	0.899	1.333	0.032	0.042	0.484
9	0.052	0.238	0.993	1.771	0.053	0.055	0.069	0.031	0.212	1.023	1.788	0.022	0.022	1.431
12	0.055	0.249	1.070	1.926	0.056	0.059	0.067	0.027	0.188	1.116	1.933	0.020	0.016	1.930
	Cell Viability: Relative Cell Growth[d]													
growth time (h)[c]														
16	*10*	*10*	*10*	*10*	*10*	*10*	*0*	*0*	*10*	*10*	*10*	*0*	*2*	*10*
16	10	10	10	10	10	10	1	0	10	10	10	0	2	10
30							*10*	*0*[e]				*10*	*10*	
30							10	0[e]				10	10	

[a] An overnight culture of B834(DE3)/pLR102 was grown in $M9_{Glu,amp}$ supplemented with 0.5 mM L-met. The cells were collected, washed and resuspended in $M9_{Glu,amp}$, and used to inoculate aliquots of $M9_{Glu,amp}$ supplemented with L-Met, L-TFM, or both as indicated (performed in triplicate) and samples incubated at 37 °C. The absorbance at 600 nm was recorded at the indicated times. [b] Values represent the average ± 0.01. [c] Following the initial 12 h growth, cells from each sample were washed and diluted 1:100 into $M9_{Glu}$ or $M9_{Glu,amp}$ (italicized growth time) each supplemented with 0.5 mM L-Met and grown at 37 °C. At given times, cultures were observed and cell growth was assessed according to the turbidity of the culture. [d] Based on a scale of 0 to 10, 0 representing a clear culture (no observable growth) and 10 representing a heavily turbid culture. [e] No growth observed following 48 h incubation.

For Low Levels of Incorporation. Aliquots (typically 10−50 mL) were supplemented with 1.0 mM L-TFM, 0.75 mM IPTG, and L-Met ranging in concentration from 0.1 nM to 1.0 mM in 10-fold increments and grown for 9.5 h at 37 °C. Following removal of a 0.5 mL aliquot for SDS−PAGE analysis, cells were collected, resuspended in 3−6 mL 50 mM potassium phosphate (pH 7), and disrupted by sonication. The lysates were filtered and applied to a S-Sepharose Fast Flow HR 5/5 column (Pharmacia) equilibrated with 50 mM potassium phosphate (pH 7). A linear gradient to 0.25 M KCl in the same buffer was implemented and active fractions collected and lyophilized. Samples were resuspended in 100 μL of Milli-Q water, filtered, and further purified by reverse phase chromatography (using gradient 1) as described below and subjected to ESMS.

Effect of TFM on Cell Growth. An overnight culture (4 mL) of *E. coli* B834(DE3) harboring pLR102 was grown in $M9_{Glu,amp}$ supplemented with 0.5 mM L-Met. The cells were collected and washed with $M9_{Glu,amp}$ and resuspended in 2 mL of the same media. A 40 μL aliquot of the cell suspension was used to inoculate 4 mL samples of $M9_{Glu,amp}$ supplemented with L-Met, L-TFM, or both as indicated in Table 1 (performed in triplicate) and cultures were incubated at 37 °C. Cell growth was monitored over time by following the absorbance at 600 nm for each culture. Following the initial 12 h incubation period, cells from 0.5 mL of each sample culture were collected, washed, and resuspended in 0.5 mL of $M9_{Glu}$, and 10 μL of each was then subcultured into 1.0 mL $M9_{Glu}$ or $M9_{Glu,amp}$ each supplemented with 0.5 mM L-Met and grown at 37 °C. Cultures were observed with time and culture growth was assessed according to the turbidity of the culture.

NMR Spectroscopy. NMR data were obtained at 376.3 MHz on a Varian Unity spectrometer fitted with a 5 mm dual broadband probehead with the proton coil tuned to fluorine. Standard undecoupled spectral parameters were 27 740 Hz spectral width, 32K data points zero filled to 64K, 10 μs pulse width, 0.577 s acquisition time with a 0.5 s relaxation delay. The first six points of the FIDS were corrected by linear prediction before Fourier transformation with a line broadening of 0.5 Hz. Unless otherwise stated, all spectra were recorded at 22 ± 0.5 °C. The ^{19}F spin−lattice relaxation times (T_1) were estimated by the inversion recovery method. Phase-sensitive 2D exchange spectra (EXSY) were accumulated (512 data points in t_2 for each of 64 t_1 values, 180 scans per increment) with mixing times from 0.1 to 0.9 s (Jeener et al., 1979). Samples were measured using a coaxial insert containing CD_3OD (as the lock solvent) and $CFCl_3$ as an external frequency standard (referenced to 0.00 ppm). The insert was placed into a standard 5 mm NMR tube containing the sample. Buffer composition and TFM-LaL concentrations for NMR studies are given in figure and table legends. Chitopentaose [$(GlcNAc)_5$], used for sugar binding experiments, was introduced directly into samples in the NMR tube to the desired concentrations (with the assumption that the volume did not change), and the tube was gently agitated to obtain complete and uniform dissolution. The error for the integrations of individual resonances was estimated by measuring the ratio of each resonance to the other three from the same spectrum and comparing these ratios to the corresponding ones from other spectra (four spectra each of high- and low-level TFM-LaL were compared). In this manner, the average error was estimated to be no greater than 10%. The standard deviation in ^{19}F chemical shift measurements was ±0.02 ppm based on three independent measurements of the same sample of high-level TFM-LaL.

Mass Spectrometry. Electrospray mass spectrometry was performed on a Fisons VG Quatro II triple-quadrupole mass spectrometer using a delivery solvent of 1:1 H_2O/CH_3CN (containing 0.1% TFA or acetic acid) and data analyzed with the Masslynx software. Multiply charged electrospray spectra were subjected to the MaxEnt algorithm to produce true molecular mass spectra with associated errors. MaxEnt data are quantitative and bar spectrum proportional to the area under individual peaks were created to estimate the relative quantities of the components in each spectrum.

Reverse Phase Chromatography. HPLC was performed using a Waters 625-LC and 994 detector over a 2.0 mm × 15 cm Delta Pak C_{18} column (Waters). The column was eluted at 0.2 mL/min employing linear gradients from buffer A (0.1% TFA in H_2O) to buffer B (0.1% TFA in CH_3CN) with detection at 215 nm. Different gradients were employed for the following desired applications.

Gradient 1: Desalting of Protein Samples for ESMS. The column was equilibrated with 80% buffer A, and the gradient

was 0−10−35 min, 20−40−65% buffer B. Both LaL and TFM-LaL eluted at approximately 18−20 min and peaks were collected from base line to base line and used directly for ESMS.

Gradient 2: Separation of High-Level TFM-LaL Species. The column was equilibrated with 75% buffer A, and the gradient was 0−10−20−80 min, 25−35−37−43% buffer B.

Molecular Modeling and NMR Calculations. Gas phase *ab initio* energy calculations and geometry optimizations on ethyl methyl sulfide and ethyl trifluoromethyl sulfide were performed at the RHF/6-31G*//RHF/6-31G* and MP2/6-31G**//RHF/6-31G* levels using either Hyperchem Version 4.5 or Gaussian 94W (RevC.3; Frisch et al., 1995). Frequency calculations at the RHF/6-31G* level were utilized to confirm the nature of the stationary points and to obtain zero-point vibrational energies (scaled by a factor of 0.89; Hehre et al., 1986). Calculated van der Waals and solvent-accessible volumes were calculated under the QSAR option in ChemPlus (Hypercube Inc., Waterloo, ON) based on the Grid method (Grid = 50) described by Bodor and co-workers (1989) using the atomic radii suggested by Gavezotti (Gavezotti, 1983). Log *P* values were obtained using atomic parameters in ChemPlus based on the work of Viswanadhan and co-workers (1989). Predicted gas phase ^{19}F isotropic magnetic shieldings in ppm were calculated for $CFCl_3$, $CF_3SCH_2CH_3$ (trans configuration), and the sulfoxide $CF_3S(O)CH_2CH_3$ at the RHF/6-311+G(d,p)//RHF/6-31G* level utilizing the GIAO method (Wolinski et al., 1990) as implemented in G94(RevC.3). The predicted ^{19}F chemical shifts were then determined for the various compounds by setting the calculated ^{19}F magnetic shielding for $CFCl_3$ equal to zero.

Miscellaneous Methods. LaL and TFM−LaL activities and inhibition studies were measured by the turbidimetric assay using chloroform treated *E. coli* cells as described previously (Duewel et al., 1995). SDS−PAGE was performed with a PhastSystem (Pharmacia) or Mini-Protean II unit (Bio-Rad).

Protein Determinations. The extinction coefficient at 280 nm (ϵ_{280}) for LaL was determined to be 31 712 ± 210 M^{-1} cm^{-1} on the basis of the method of Gill and von Hippel (1989) and the amino acid sequence (Sanger et al., 1982). The ϵ_{280} was assumed to hold for the different preparations of TFM-LaL.

RESULTS

Synthesis of L-*TFM*. The synthesis of D,L-TFM was first reported by Dannley and Taborsky (1957) but suffered from low overall yield (11% in five steps) with the generation of a racemic product. The current method is based on our previous protocols (Houston & Honek, 1989; Houston, 1992). In the presence of methoxide, *N*-acetyl-D,L-homocysteine thiolactone is converted to the thiolate *in situ* which is reacted with excess trifluoromethyl iodide giving the fully protected racemic amino acid. Alkylation of the thiolate proceeds via a radical mechanism and requires substantial long wave UV irradiation. Saponification of the methyl ester followed by resolution and deprotection with acylase generated the desired L isomer of *S*-(trifluoromethyl)homocysteine in an overall yield of 33% (Scheme 1).

Overproduction of LaL. By using PCR, we have prepared the plasmid pLR102 which gave strong overproduction of LaL. Purification of LaL from BL21(DE3)/pLR102 grown

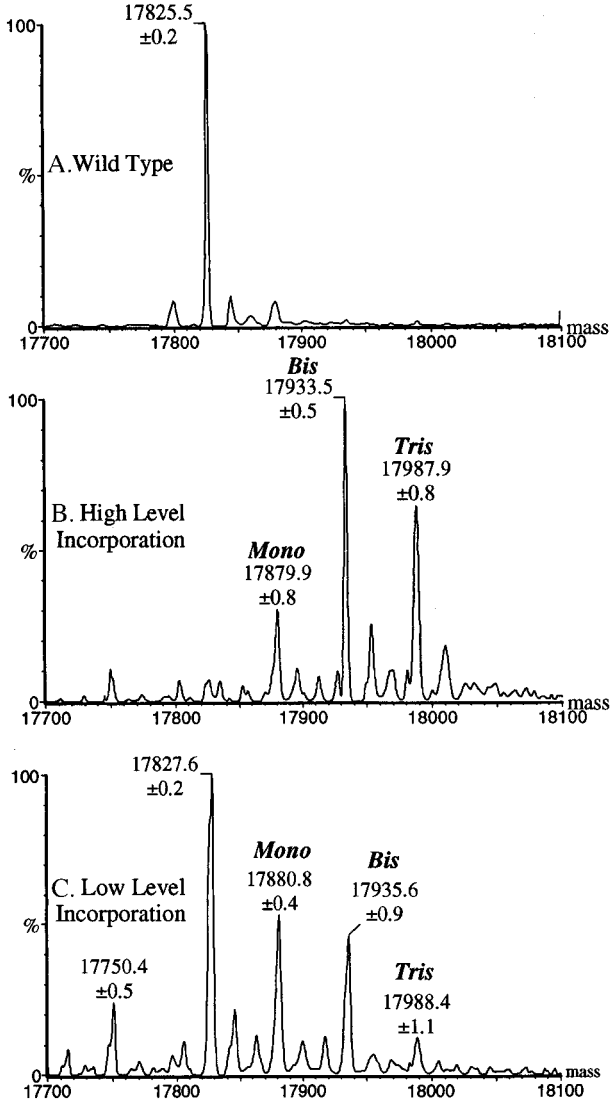
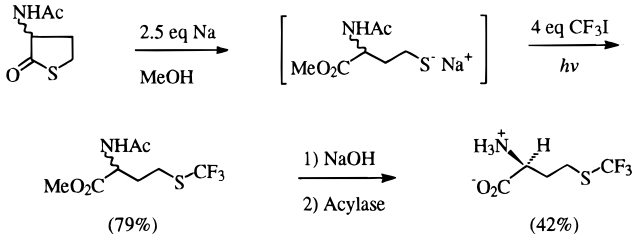


FIGURE 1: Reconstructed electrospray mass spectra of purified lysozymes indicating observed molecular masses (Da). (A) Wild type lysozyme. (B) Lysozyme labeled with 70% TFM. (C) Lysozyme labeled with 31% TFM. The labels mono, bis, and tris refer respectively to lysozyme labeled with one, two, or three TFM residues. The calculated molecular masses (Da) are as follows: wild type, 17 825.2; mono-labeled, 17 879.2; bis-labeled, 17 933.2; tris-labeled, 17 987.1.

Scheme 1



in enriched medium (Luria-Bertani) or B834(DE3)/pLR102 grown in minimal medium containing L-methionine (>0.2 mM) gave consistently 25−30 mg of purified protein per liter of cell culture. Wild type lysozyme has a calculated molecular weight of 17 825.2 Da, and the expected mass of the purified enzyme was detected from ESMS analysis (Figure 1A).

Optimal Growth Conditions To Produce TFM-LaL. The analogue L-TFM will not support growth of the methionine

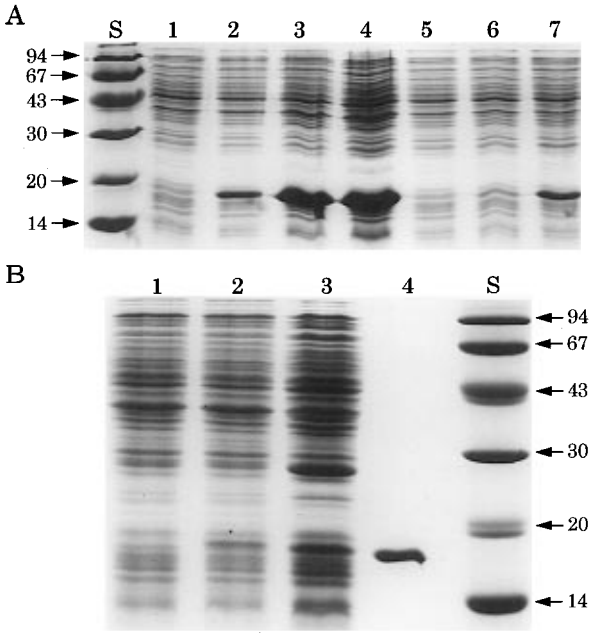


FIGURE 2: Comparison of the expression efficiency of LaL and TFM−LaL under various conditions. *E. coli* B834 harboring pLR102 was grown in $M9_{Glu,amp}$ (containing 0.1 mM L-Met) to an absorbance of 0.65 at 600 nm, cells were collected, washed and reintroduced into one-half the initial growth volume of $M9_{Glu,amp}$. Cultures were then treated as described in the following text and aliquots were subjected to 15% SDS−polyacrylamide gel electrophoresis analysis. (A) Cells subjected to a 4 h induction period in the presence of 0.01, 0.1, or 1.0 mM of L-Met (lanes 2−4) or of L-TFM (lanes 5−7). Lane 1 represents cells induced with no methionine supplement. (B) Cells subjected to 9.5 h of growth without induction or methionine supplement (lane 1), with induction and no methionine supplement (lane 2) or in the presence of 1.0 mM TFM (lane 3). Lane 4 is purified high-level TFM-LaL. Lanes S are molecular mass standards (in kilodaltons).

auxotroph (Table 1 and discussed below). For this reason, *E. coli* B834(DE3)/pLR102 was initially grown in minimal medium containing 0.1 mM L-methionine to mid-exponential growth. Cells were collected and washed to remove residual methionine from the medium and reintroduced into one-half the original volume of fresh medium supplemented solely with L-TFM or in combination with L-methionine and induced with IPTG.

To obtain high incorporation levels of TFM into LaL, cultures were induced in the presence of 1.0 mM of the fluorinated analogue for 9.5 h. Care was taken during the wash procedure to ensure thorough removal of methionine from the media. As discussed below, induction in the presence of methionine at concentrations as low as 10 μM will considerably reduce TFM incorporation. LaL expression levels were analyzed for media supplemented with various concentrations of L-TFM ranging from 0.01 to 5 mM and duration of induction period. Maximum protein expression was obtained at 1.0 and 2.0 mM L-TFM, and since the analogue appears to be toxic at higher concentrations, 1.0 mM was chosen as the supplement concentration. Interestingly, L-TFM at 5.0 mM resulted in lower levels of protein expression most likely due to the bactericidal effect of the analogue at this concentration (Table 1). Protein synthesis, in general, was found to increase with time (18 h > 9.5 h > 4 h) in the presence of the inducer, IPTG, and this phenomenon was found to occur regardless of the addition of exogenous methionine (see lane 1, Figure 2A, and lane 2, Figure 2B). These results suggest that during prolonged growth, the auxotroph is able to recycle endogenous sources of methionine from protein degradation or from salvage of N-terminal methionine residues by methionine aminopeptidase. This new supply of methionine would compete with L-TFM to reduce the relative incorporation of the analogue into each of the methionine positions (positions 1, 14, and 107) in LaL. For these reasons, an induction period of 9.5 h was chosen for further study.

We define mono-, bis-, and tris-labeled LaL species as enzyme in which one, two, or three of the three methionine positions have been replaced with TFM respectively. The ESMS spectrum of TFM−LaL prepared under high-incorporation conditions clearly demonstrates that TFM has been incorporated at each of the locations normally occupied by methionine in LaL (Figure 1B). In addition, partial sequencing of the N-terminus of high-level TFM-LaL (results not shown) has established that Met1 and Met14 have been substituted with TFM. With the assumption that the ionization of each species is the same, the spectrum can be interpreted quantitatively. On the basis of the areas under the peaks, the relative amounts of tris:bis:mono:*wt* are 1:1:0.5:0.1. Statistically, there are three different mono- and three different bis-labeled species and only one tris-labeled species possible. Considering that the individual species present represent 33% (mono), 66% (bis), and 100% (tris) incorporation respectively and given the relative quantities of each, the overall extent of incorporation can be calculated to be approximately 70%. In another preparation of high-level TFM-LaL, incorporation was found to be 74% by the same analysis. Between 1 and 2 mg of purified lysozyme (lane 4, Figure 2B) is obtained per liter of culture prepared under high-incorporation conditions.

In order to establish conditions that would generate lower levels of incorporation, samples for ESMS analysis were prepared by varying the concentration of L-methionine during induction from 0.1 nM to 1 mM (each containing 1.0 mM L-TFM). Below 10 μM L-methionine, the ESMS spectra of TFM−LaL essentially resembled that shown in Figure 1b. Increasing methionine concentrations led to a proportional decrease of tris-labeled LaL with concomitant increase in both *wt* and mono-labeled enzyme. A marked difference in the spectra was observed between 10 and 100 μM L-methionine and therefore 20, 40, 60, and 80 μM concentrations were also investigated. L-Methionine supplements above and including 60 μM resulted in no appreciable levels of species containing TFM as the spectra were very similar to that of the wild type enzyme. L-Methionine supplements of 10, 20, and 40 μM resulted in comparable detectable labeled species and 20 μM was chosen for further investigation. As seen in Figure 1c, TFM−LaL prepared under low-level incorporation conditions generated each of tris-, bis-, and mono-labeled species with *wt* predominating in relative amounts of 0.16:0.5:0.6:1, respectively. As described above, the extent of incorporation is calculated to be approximately 31%, and 15 mg/L of purified protein is obtained. The precision of calculating incorporation levels by ESMS was assessed by a triplicate determination of low-level TFM−LaL, which gave 30.5% ± 0.7%.

Effect of TFM on Cell Growth. In our efforts to optimize conditions for the incorporation of L-TFM into proteins, we were also interested in the effects of the analogue on cell growth in the absence of IPTG induction. As is expected, L-methionine is required to permit growth of the *E. coli*

Table 2: ^{19}F NMR Data for TFM-LaL

resonance[a]	T_1 (s)[b] high[e]	T_1 (s)[b] low[f]	percentage of total integrated area[c] high	percentage of total integrated area[c] low	chemical shift[d] high	chemical shift[d] low
A	0.80 ± 0.10	0.84 ± 0.04	33.9 ± 1.2	37.2 ± 1.3	−39.32	−39.36
B	0.38 ± 0.03	0.40 ± 0.01	33.5 ± 1.3	33.3 ± 1.8	−39.82	−39.88
C	0.51 ± 0.17	0.54 ± 0.04	11.8 ± 0.6	19.1 ± 0.5	−39.99	−40.04
D	0.50 ± 0.07	0.59 ± 0.06	20.8 ± 1.1	10.4 ± 1.5	−40.11	−40.16

[a] Refer to Figure 3 for assignments. [b] ^{19}F spin−lattice relaxation times determined by the inversion recovery method. [c] Determined by comparing the integral of each resonance to the sum of all resonance integrals in the same spectrum ($n \geq 3$). [d] Referenced to $CFCl_3$ (0.00 ppm) ± 0.02 ppm. [e] High-level TFM−LaL labeled with 70% TFM. Protein concentration was 0.27 mM in 55 mM potassium phosphate, pH 7.0. [f] Low-level TFM−LaL labeled with 31% TFM. Protein concentration was 1.53 mM in 50 mM potassium phosphate, pH 7.0.

auxotroph B834(DE3) harboring pLR102, with the total growth increasing with L-Met concentration as a function of time (Table 1). Clearly, L-TFM will not support cell growth. At concentrations examined for L-TFM of 1.0 mM and below, culture growth is minimal and similar to the control where no methionine supplement was included. L-TFM at 5.0 mM did appear to have an adverse affect on growth when compared to the control (Table 1). Cell growth was also influenced in those cultures supplemented with L-TFM together with L-Met. At high ratios of L-TFM/L-Met (5.0 mM/0.01 and 0.05 mM) cell growth is effectively inhibited. An adverse effect on growth is also observed in cultures containing concentrations of L-TFM/L-Met of 1.0 mM/0.01 mM and 5.0 mM/0.5 mM when compared to those grown with L-Met alone at these levels. Cell growth in cultures containing 1.0 mM L-TFM and 0.05 or 0.5 mM L-Met was not affected. It is apparent that L-TFM does impart a dose dependent toxicity to cell growth when the concentration of the analogue is substantially greater than that of L-Met. This inhibition may be a consequence of the analogue interfering with the normal metabolic utilization of methionine or with other cellular processes in some manner.

Not only are high concentrations of L-TFM detrimental to cell growth, but L-TFM also appears to affect the viability of cells. As indicated in Table 1, cells grown for 12 h in the presence of 1.0 mM L-TFM alone and 5.0 mM L-TFM with 0.01 and 0.05 mM L-Met prior to subculturing into fresh media containing 0.5 mM L-Met showed a substantial lag period (>16 h) before reversal of growth inhibition was observed. This lag period extended to greater than 48 h for cells initially grown in the presence of only 5.0 mM L-TFM. No difference in cell viability was noted when cells were subcultured into media with or without ampicillin, suggesting that lack of growth in the former media can not be attributed to plasmid instability. In the case of *Saccharomyces cerevisiae*, 10 μM L-TFM produced an indefinite lag period which could be reversed by methionine or *S*-adenosylmethionine (Colombani et al., 1975).

^{19}F NMR Spectra of TFM−LaL. Lysozyme contains three methionines at positions 1, 14, and 107; however, four resonances are observed in the ^{19}F NMR spectra of TFM-labeled lysozyme prepared under high-level (70%) and low-level (31%) incorporation conditions (Figure 3). Our evidence suggests that one of the three methionines gives rise to the resonances C and D and that the relative intensity of each of these two peaks is dependent on the extent of overall TFM incorporation into the protein (discussed below). In both high- and low-level TFM-LaL, the resonances span a chemical shift range of approximately 0.9 ppm. The resonances are rather sharp with the line widths at half height

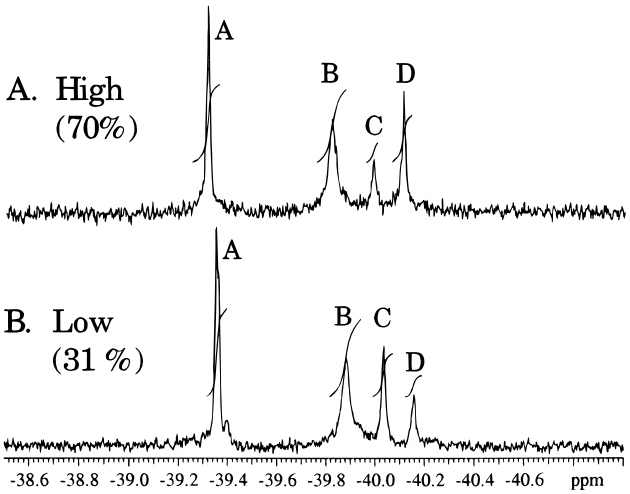


FIGURE 3: ^{19}F NMR spectra of high- and low-level incorporated TFM-labeled lysozymes. The approximate extent of incorporation determined from ESMS spectra are indicated. No additional resonances were observed over the chemical shift range of −66 to 8 ppm as referenced to $CFCl_3$ (0.00 ppm). (A) Lysozyme labeled with 70% TFM. Protein concentration was 270 μM in 55 mM potassium phosphate, pH 7.0. A total of 2000 scans were acquired. (B) Lysozyme labeled with 31% TFM. Protein concentration was 1.53 mM in 50 mM potassium phosphate, pH 7.0. A total of 1500 scans were acquired.

measured to be approximately 7−9 Hz for peaks A, C, and D and 15 Hz for resonance B in both spectra. Since the line shape of the resonances is not affected at the two different protein concentrations studied (0.27 and 1.53 mM for high- and low-level TFM−LaL respectively), no appreciable self-aggregation of TFM−LaL is suggested at the concentrations used for these NMR studies. Other NMR data are summarized in Table 2. The small upfield shift (≤0.06 ppm) noted for resonances in low-level TFM-LaL when compared to the analogous ones for high-level TFM−LaL may arise from the differences in protein and buffer concentrations of the two samples (Table 2).

Although the resonances have not yet been assigned, they are still useful as probes. In both high- and low-level TFM−LaL, resonances B and C/D show very modest upfield shifts on addition of $(GlcNAc)_5$ that increase with increasing sugar concentration while resonance A remains relatively unchanged (Table 3). These results are consistent with our previous observations of a slight alteration in two of the three ^{13}C resonances upon binding of $(GlcNAc)_5$ to [*methyl*-^{13}C]-Met-labeled LaL (Duewel et al., 1995). The spectra of high-level TFM-LaL in the presence of $(GlcNAc)_5$ are shown in Figure 4. Upon sugar binding resonance B not only exhibits the largest upfield shift but also displays substantial line broadening. The line width at half height increases from

Table 3: Effect of $(GlcNAc)_5$ Binding on the ^{19}F NMR Resonances of TFM−LaL

	$\Delta\delta$ (ppm) resulting from $(GlcNAc)_5$ binding[b]					
	2 mM		5 mM		10 mM	
resonance[a]	high[c]	low[c]	high	low	high	low
A	−0.01	nd[d]	−0.02	−0.02	−0.03	−0.02
B	−0.06	nd	−0.11	−0.09	−0.14	−0.11
C	−0.04	nd	−0.06	−0.05	−0.07	−0.07
D	−0.03	nd	−0.05	−0.05	−0.06	−0.06

[a] Refer to Figure 4 for assignments. [b] Differences relative to chemical shifts given in Table 2 for unliganded TFM−LaL. The negative sign (−) indicates a shift to higher field. [c] Defined in the legend for Table 2. [d] nd, not determined.

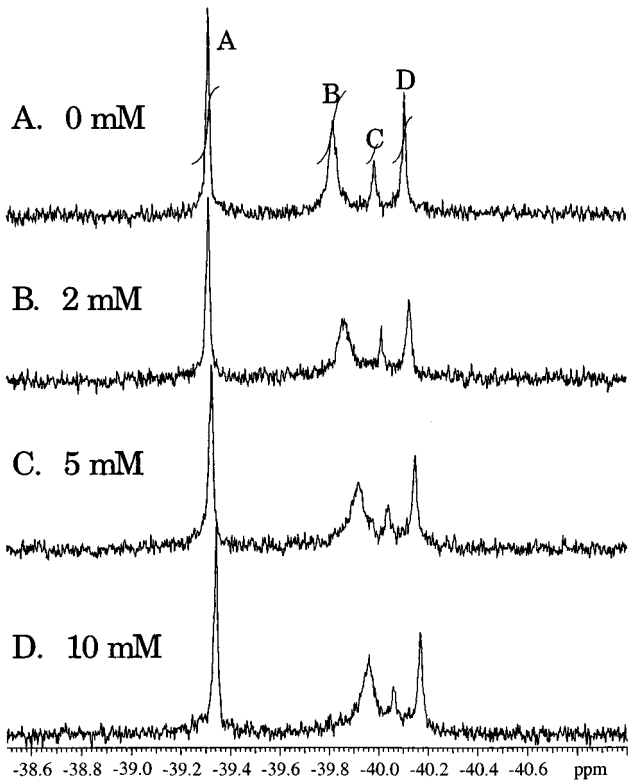


FIGURE 4: Effect of $(GlcNAc)_5$ binding on the ^{19}F NMR resonances of lysozyme labeled with 70% TFM. All samples contained 270 μM TFM-LaL and 55 mM potassium phosphate, pH 7.0. A total of 2000−3000 scans were acquired. The samples also contained (A) no $(GlcNAc)_5$, (B) 2 mM $(GlcNAc)_5$, (C) 5 mM $(GlcNAc)_5$, and (D) 10 mM $(GlcNAc)_5$.

15 Hz [in the absence of $(GlcNAc)_5$] to approximately 26−30 Hz in the presence of $(GlcNAc)_5$ at each concentration studied. The general appearance (peak line widths) of the spectra of low-level TFM−LaL upon sugar binding is identical to that shown in Figure 4 except that the intensities of resonances C and D are reversed (data not shown).

The ^{19}F NMR results obtained for peaks C and D suggest that they arise from the same positional methionine. If the data for these peaks (Tables 2 and 3) are examined independently for both high- and low-level TFM−LaL, then within experimental errors (i) the integral sum of C/D approximately equals that of A or B, (ii) resonances C and D have equivalent spin−lattice relaxation times which differ from A and B, and (iii) resonances C and D undergo corresponding chemical shift changes upon $(GlcNAc)_5$ binding. Furthermore, the results indicate that TFM is incorporated equally well at the three Met positions since resonances A and B each represent approximately one-third of the total integrated area while the integral sum for C and D account for the remaining one-third (Table 2).

DISCUSSION

In our ongoing studies of methionine biochemistry, we have pursued the incorporation of L-TFM into bacteriophage lambda lysozyme. This lysozyme is a late-gene product of the λ bacteriophage and, unlike many lysozymes, cleaves bacterial peptidoglycan to 1,6-anhydro-containing oligosaccharides (Taylor et al., 1975). Our previous work on this enzyme utilized differential scanning calorimetry, substrate inhibition studies, and [*methyl*-^{13}C]methionine NMR labels to provide evidence for the direct binding to and inhibition of LaL by chitooligosaccharides (Duewel et al., 1995).

This account constitutes the first characterization of an enzyme into which TFM has replaced methionine residues. There has previously been only indirect evidence of TFM incorporation into TCA-insoluble protein fractions from *S. cerevisiae* (Colombani et al., 1975) but no detailed characterization of a TFM-containing protein has been reported to date. Substitution of Met by TFM at positions 1 and 14 in high-level TFM-LaL has been confirmed by N-terminal sequencing and, taken together with analysis of TFM-LaL by ESMS and ^{19}F NMR, is a clear indication that incorporation has occurred at each of the three Met positions (positions 1, 14, and 107) in LaL.

The incorporation of TFM has also provided some additional information on the specificities of the enzymes involved in protein biosynthesis. Methionyl-tRNA synthetase is responsible for the acylation of both the elongator $tRNA^{Met}$, for insertion of Met into internal peptide linkages, and of the initiator $tRNA_i^{fMet}$. The crystal structure of *E. coli* methionyl-tRNA synthetase has been determined (Brunie et al., 1990) and methionine has been modeled into the active site (Kim et al., 1993). In the proposed model, the methyl group of Met is positioned between the side chains of Phe197 and Trp305. The interactions between methionyl-tRNA synthetase and TFM must therefore be of sufficient nature to accommodate the trifluoromethyl group and permit the analogue to proceed through the synthetic pathway to form TFM-$tRNA^{Met}$ and TFM-$tRNA_i^{fMet}$.

Eubacterial translation initiation also involves formylation of Met-$tRNA_i^{fMet}$ to fMet-$tRNA_i^{fMet}$ by a N^{10}-formyltetrahydrofolate dependent transformylase (RajBhandary, 1994). If indeed formylation of TFM-$tRNA_i^{fMet}$ does occur, the ensuing deformylation by the endogenous deformylase (Meinnel & Blanquet, 1995) must also proceed since ESMS analysis of TFM−LaL did not give any evidence of formylation of the mature proteins. Finally, results from integration of the resonances obtained from the ^{19}F NMR studies of TFM−LaL (Table 2) suggest that all three methionine locations incorporate L-TFM equally well.

The characterization of TFM−LaL clearly illustrates the ability of the biosynthetic system to recognize and incorporate L-TFM into methionine locations. However, it is also evident that protein expression is substantially reduced when L-TFM serves as the methionine source. We have prepared TFM-labeled LaL at incorporation levels of 31% and 70%. High levels of incorporation are obtained by induction of cells in the presence of 1.0 mM L-TFM but is accompanied by low protein expression. It is clearly seen (Figure 2A) that the amount of LaL produced is considerably less when

induction occurs in the presence of L-TFM (lanes 5−7) instead of L-Met (lanes 2−4). Lower levels of incorporation with concomitant increase in protein yields are obtained when protein expression is conducted in media containing both L-TFM (1.0 mM) and L-Met (20 μM). This is also reflected in the amount of purified wt LaL obtained under L-Met-enriched conditions (25−30 mg/L) as compared to that obtained for high-level TFM−LaL (1−2 mg/L) and low-level TFM−LaL (15 mg/L). The decreased expression may be the result of inefficient utilization of L-TFM in the translation process. Also, there may be a reduced intracellular availability of L-TFM due to a reduced affinity of the transport system (Kadner, 1977) for the fluorinated analogue.

The toxic effect of L-TFM on cell growth was initially reported by Zygmunt and Tavormina (1966) for a variety of microorganisms. These workers observed that 100 μg/mL (0.49 mM) of D,L-TFM failed to inhibit the growth of *E. coli* in an enriched medium; however, substantial inhibition resulted using a minimal medium. In the latter instance, it appears that *de novo* biosynthesis of methionine or its intracellular utilization may be influenced by TFM, and if methionine is present in the medium, this effect is diminished. These results support our observations of growth inhibition with the *E. coli* methionine auxotroph B834(DE3)/pLR102. In *S. cerevisiae*, TFM has been shown to cause a dramatic decrease in the intracellular free pool of methionine which correlated with inhibition of growth presumably due to its function as a repressor of the biosynthesis of this amino acid (Colombani et al., 1975). This may also be the case in *E. coli*, however we have not fully explored the mechanism of growth inhibition by this analogue.

As this approach may be of general use to the study of protein−ligand and protein−protein interactions by ^{19}F NMR spectroscopy, it is interesting to explore some of the fundamental properties of the side chain of TFM compared to that of L-methionine. The thiobutane side chain of L-methionine is important for a number of biochemical properties. The side chain itself is more flexible than those of related amino acids such as leucine and isoleucine and this has been ascribed to the longer CH_3S-CC bond which reduces the steric repulsions usually seen for all carbon alkyl chains (Gellman, 1991). In fact, the χ_3 torsion angles for methionine residues in proteins show very little preference for any particular value(s) (Janin et al., 1978). Experimental evidence over the years, based on electron diffraction (Oyanagi & Kuchitsu, 1978) and microwave vibrational spectroscopy (Nogami et al., 1975), has indicated that the gauche and trans conformations of ethyl methyl sulfide are close in energy, and in fact both are found in the gas and liquid phases. Recently Durig and co-workers (1991) have reported that in fact the trans conformation may be slightly more stable in the gas phase than the gauche by approximately 0.38 kcal/mol. This flexibility of the thiobutane moiety permits numerous conformations to be taken up by the methionine side chain. In addition, the sulfur atom in methionine is a polarizable atom and this has been suggested to play a role in sequence independent recognition of nonpolar protein surfaces.

In the case of TFM, one needs to consider the possible conformational and electronic effects that the incorporation of fluorine has on the methionine structure. In terms of conformation, we have utilized *ab initio* calculations to study ethyl methyl sulfide and ethyl trifluoromethyl sulfide as models for Met and TFM. Since methionine is usually encountered in the hydrophobic interior of most globular proteins, gas phase calculations may in fact be appropriate indicators for this system.

High level *ab initio* calculations with geometry optimizations at the //RHF/6-31G* level have been shown to rather accurately predict the experimental stabilities and structures of sulfides such as ethyl methyl sulfide (Durig et al., 1991; Markham & Bock, 1995). At the RHF/6-31G*//RHF/6-31G* level, both the trans conformers of ethyl methyl sulfide and ethyl trifluoromethyl sulfide are more stable than their gauche conformers by 0.41 kcal/mol and 0.39 kcal/mol respectively. However, if electron correlation is taken into account utilizing the MP2/6-31G**//RHF/6-31G* level, the gauche conformer of ethyl methyl sulfide and ethyl trifluoromethyl sulfide are predicted to be only slightly more stable than the trans by 0.002 and 0.27 kcal/mol, respectively. It appears that even in the fluorinated case, the trans and gauche conformers are very close in energy and are probably both sampled and energetically attainable in TFM. The dihedral angles for the gauche conformations of ethyl methyl sulfide and ethyl trifluoromethyl sulfide at this level of calculation are 70.5° and 80.8°, respectively, indicating an "opening up" of the gauche structure in the case of TFM which may contribute to the overall properties of a TFM-containing protein such as LaL.

The Mulliken charge on the sulfur atom in TFM (0.153 e) versus Met (0.113 e) and the highest-occupied molecular orbital (HOMO) energy for this analogue (−10.38 eV) compared to the methionine (−9.03 eV) suggest a reduced nucleophilicity of the sulfur atom in TFM. We have established that TFM exhibits reduced reactivity with both hydrogen peroxide and cyanogen bromide compared to Met (results not shown). However, it should be noted that the CF_3S- moiety itself does not entirely preclude the possibility of its ligation to metal centers. For example, the crystal structure of a CF_3-sulfide ligand to platinum in *cis*-dichloro-[1,2-bis((trifluoromethyl)thio)propane]platinum(II) indicates ligation of the sulfur to platinum (Manojlovic-Muir et al., 1977) and hence TFM may indeed exhibit ligation in proteins where normally methionine is encountered. In the case of fluorinated aromatic amino acids such as those utilized for Trp, Phe, and Tyr, the electron density of the aromatic π systems will also be affected although this has not detracted from their highly successful application as ^{19}F NMR probes.

Van der Waals and solvent accessible volumes based on //RHF/6-31G* level geometries for ethyl trifluoromethyl sulfide were calculated to be 87.5 and 350 Å^3, respectively, and are somewhat larger than the values calculated for ethyl methyl sulfide (80.6 and 327 Å^3, respectively). Therefore, steric effects may play a role in a protein with incorporated TFM. In addition, the CF_3 group is hydrophobic and the log *P* value for ethyl trifluoromethyl sulfide was calculated to be 2.73 compared to 0.80 for ethyl methyl sulfide. This predicted hydrophobicity of a TFM residue is exemplified experimentally by the increase in retention time observed on reverse-phase HPLC by LaL molecules containing higher levels of TFM. It was observed that samples of TFM−LaL desalted by reverse phase chromatography in preparation for ESMS analysis tended to produce somewhat broader peaks than did *wt* LaL employing a sharp elution gradient (gradient 1). When TFM−LaL was chromatographed using a much shallower gradient (gradient 2), it was possible to obtain

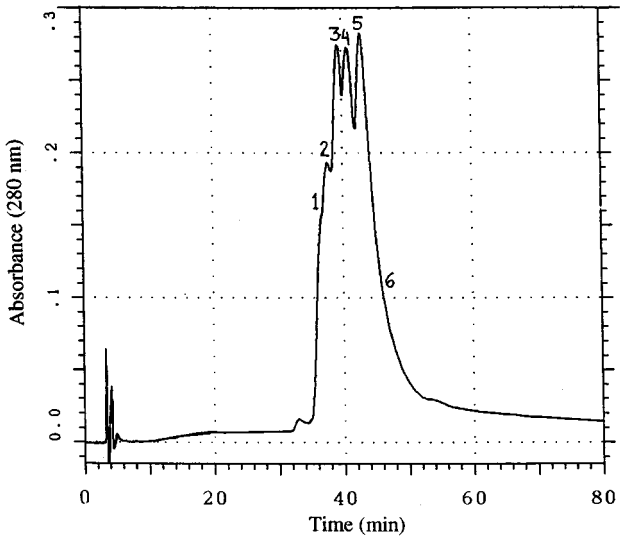


FIGURE 5: Reverse phase chromatogram of high-level TFM-LaL. Chromatographic conditions are outlined under Materials and Methods using gradient 2. Fractions (1−6) were collected and subjected to ESMS to identify the respective components present. The major (and minor in parentheses) species detected in each fraction were: (1) *wt* LaL and mono-TFM−LaL (Met$^-$ *wt* LaL); (2) mono-TFM−LaL (bis-TFM−LaL, Met$^-$ mono-TFM−LaL); (3) bis-TFM−LaL (Met$^-$ mono-TFM−LaL); (4) bis-TFM−LaL (Met$^-$ bis-TFM−LaL); (5) tris-TFM−LaL (bis-TFM−LaL); and (6) tris-TFM−LaL. The prefix Met$^-$ denotes a species of mass corresponding to loss of a single Met residue.

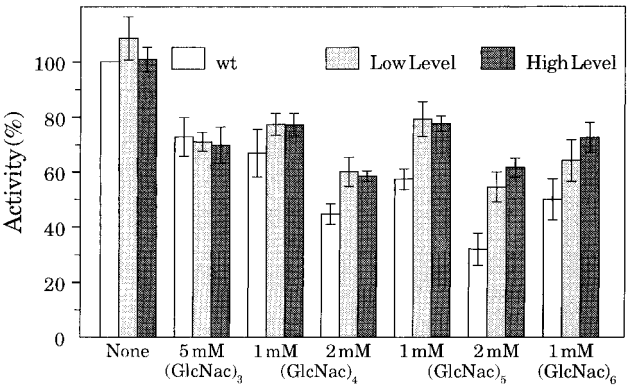


FIGURE 6: Comparison of the activity and inhibition of *wt* and TFM-LaL. A turbidimetric assay utilizing chloroform treated *E. coli* cells in 50 mM phosphate buffer (pH 7) was employed to monitor the bacteriolytic activity of LaL. Illustrated are the activities for *wt* LaL and low-level and high-level TFM−LaL in the absence (none) or presence of chitooligosaccharides [$(GlcNAc)_n$] at the indicated concentrations.

partial separation of the component species and analyze these individual fractions by ESMS (Figure 5). Clearly, retention times on the C18 hydrophobic support increased with proportionate content of TFM, with tris-labeled TFM-LaL having the strongest interactions. The results demonstrate the increased hydrophobic character to the protein imparted by the trifluoromethyl group.

If the methionine residues are important in the hydrophobic effect for folding, the incorporation of TFM into the protein appears not to greatly affect the folding process. Incorporation of L-TFM into LaL generated catalytically active proteins that were inhibited by chitooligosaccharides (Figure 6). For lack of a suitable small molecular weight or chromogenic substrate for this enzyme, the activity of LaL was assessed turbidimetrically by monitoring its bacteriolytic (peptidoglycan degrading) activity on chloroform treated *E. coli* cells. Preparations of high- and low-level TFM−LaL exhibited relative activities comparable to *wt* lysozyme indicating that L-TFM incorporation did not result in any substantial structural alterations deleterious to enzyme activity. Compared to *wt* LaL, both high- and low-level TFM−LaL were inhibited equally well by $(GlcNAc)_3$ but appeared to show less inhibition by $(GlcNAc)_{4-6}$. Structural analysis of lysozymes from different sources have demonstrated the presence of a pronounced saccharide binding cleft comprised of five or six distinct monosaccharide subsites, which have been traditionally designated as sites A−F (Blake et al., 1967; Anderson et al., 1981). Chitooligosaccharides[$(GlcNAc)_n$] are structural mimics of peptidoglycan and were shown to bind and inhibit LaL (Duewel et al., 1995). The evidence supported stronger interactions of LaL with saccharides of increasing oligomeric size, suggesting that LaL may also possess discrete subsites which synergistically afford different modes and degrees of binding dependent on saccharide length. If such is the case, then it is reasonable to assume that length of the sugar dictates which particular sequential subsites of the protein are predominantly recruited for binding. $(GlcNAc)_3$ may bind in such a manner that none of the three methionine residues in LaL are in the vicinity of its preferred binding site and hence it interacts equivalently with *wt* and TFM−LaL. Conversely, a methionine residue(s) may be present or involved in the association with $(GlcNAc)_{4-6}$. If so, the presence of a TFM residue may interfere with proper binding and a reduced affinity reflected in decreased inhibition would be observed. In addition, the labeled enzymes generally exhibited comparable inhibition to each other although a slight difference in inhibition between the two levels may be suggested for $(GlcNAc)_5$ at 2 mM and $(GlcNAc)_6$. There is literature support for the involvement of methionine in carbohydrate binding. Methionine 108 of the arabinose-binding protein adopts specific conformations to optimize nonpolar interactions in association with its different ligands (Vermersch et al., 1991). In the case of *E. coli* exomuramidase Slt70 (Thunnissen et al., 1995) and T4 lysozyme (Kuroki et al., 1993), enzymes which like LaL must bind and cleave peptidoglycan, the crystal structures indicate the presence of a methionine residue (Met498 in Slt70 and M106 in T4 lysozyme) in or near the vicinity of their active sites.

A striking difference between the ^{19}F NMR spectra of high- and low-level TFM−LaL is the intensity of resonances C and D. We believe that one of the three TFM-incorporated positions is responsible for both of these resonances and that the respective intensities of C and D are governed by the level of TFM incorporation. Several possibilities could explain the existence of a double resonance corresponding to a single residue and each is addressed below: (1) chemical exchange of the trifluoromethyl group between different microenvironments; (2) chemically different forms of the enzyme (protein heterogeneity) producing microenvironments around the trifluoromethyl group(s) that differ amongst the particular protein species; and (3) distinct, non-interconverting populations of enzyme whose structures are dependent or influenced by the presence of either methionine or TFM at a given position. The extent of incorporation would thereby dictate the relative proportions of each population.

Chemical exchange can be attributable to rotation of the TFM side chain or conformationally different forms of the enzyme. In either case, these exchange processes must be

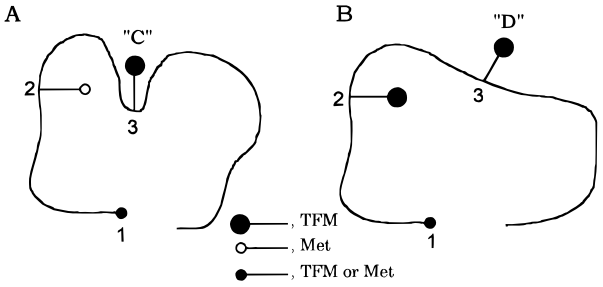
slow enough on the NMR time scale in order to observe separate resonances for a single residue (Wagner & Wüthrich, 1986). These processes could very well reflect attempts by the protein to accommodate the trifluoromethyl group. We have calculated that the volume occupied by the side chain of TFM is expected to be larger than that of L-methionine and local regions in the protein in proximity to the methionine(s) may not be sterically permissive to this substitution. Readjustment of the TFM side chain through rotation may be required and will depend on the flexibility of this side chain. It may also be possible that either the TFM may move within a flexible region of the backbone or there could be movement of an adjacent protein segment to accommodate the TFM.

We dismiss the possibility of exchange from the following observations. Firstly, spectra obtained for high incorporation TFM−LaL at 22, 35, and 45 °C were indistinguishable regarding peaks C and D. A minor sharpening of resonance B as well as a resonance corresponding to denatured protein was noted at the elevated temperatures and a spectra taken after maintaining the sample at 45° for 50 min revealed only a single sharp resonance at −39.96 ppm indicative of complete denaturation. Secondly, we also recorded phase-sensitive 2D exchange spectra (EXSY) of high-level TFM-LaL at mixing times from 0.1 to 0.9 s and found no evidence of chemical exchange for C and D.

The second possibility as to the nature of the double resonance is protein heterogeneity. A perceptive observation made by Li et al. (1989) was that doubling of resonances in the ^{19}F NMR spectra of 6-F-Trp-labeled rat cellular retinol-binding protein II was due to the presence or absence of the N-terminal methionine residue (the relative intensities of the two components of the two resonances corresponded to the relative amount of initiator methionine present). We also have evidence of N-terminal heterogeneity. ESMS of several preparations of *wt* and TFM−LaL demonstrated the presence of species corresponding to the loss of a methionine residue (results cannot indicate whether an N-terminal TFM is removed), probably arising from co- or post-translational processing by methionine aminopeptidase. For example, in Figure 1C, the peak at 17 750 Da is representative of mono-labeled TFM−LaL less the mass of Met (see also Figure 5). However, the typically low abundance of these species (<5%) could not account for the intensity ratio observed for C and D. With the intention of possibly identifying the N-terminal TFM resonance, attempts were made to utilize recombinant methionine aminopeptidase (Roderick & Matthews, 1993) to remove the N-terminal trifluoromethionine of TFM−LaL *in vitro*. However, this enzyme failed to remove the N-terminal methionine residue in *wt* LaL presumably due to either the presence of a non-optimal amino acid residue, valine, in the adjacent position or inaccessibility of the N-terminus. Hence, this approach was not extended to achieve the deletion of the N-terminal residue from LaL preparations containing TFM residues.

Although it is well known that methionine residues are susceptible to oxidation (Brot & Weissbach, 1991), we have no ESMS evidence for the occurrence of methionine oxidation in TFM−LaL which might contribute to protein heterogeneity and the origin of the C/D resonances. In addition, we have found that L-TFM itself is inert to hydrogen peroxide oxidation under conditions which are known to rapidly convert L-methionine into its sulfoxide (Brot et al., 1984). As well, gauge-independent atomic orbital (GIAO) calculations (Wolinski et al., 1990) involving the *ab initio* calculation of nuclear magnetic resonance (NMR) chemical shifts were used to determine the change in the average ^{19}F chemical shift of the trifluoromethyl group on sulfur oxidation. GIAO calculations on ethyl trifluoromethyl sulfide and its sulfoxide at the RHF/6-311+G(d,p)//RHF/6-31G* level indicated a chemical shift change from −34.8 ppm for the sulfide (relative to $CFCl_3$ as reference) to −62.8 ppm for its sulfoxide. Hence, the C/D ^{19}F NMR resonances do not originate from the presence of trifluoromethionine sulfoxide residues in LaL.

Chart 1



Proline cis-trans isomerization can also be considered to produce chemical heterogeneity in a protein and was used as a possible explanation to account for two resonances observed for 5-F-Trp-labeled D-galactose/D-glucose chemosensory receptor (Luck & Falke, 1991a). Although LaL contains five prolines, it is unlikely that the nature of the double resonance arises from isomerization since (i) lysozyme labeled with the non-perturbing probe [*methyl*-^{13}C]Met did not produce doubling of any of the three resonances observed in its [^{1}H−^{13}C]HMQC spectra (Duewel et al., 1995) and (ii) the relative occurrence of separate protein species (and therefore, relative intensities of C and D) would depend on the degree of isomerization and not, as is observed, on the extent of incorporation. One could however argue that isomerization may be influenced or even induced by the extent of TFM incorporation and therefore, the intensities of C and D could represent varying degrees of one or several isomerizations.

The third argument possibly explaining the double resonances is the existence of distinct, non-interconverting conformers of TFM-LaL whose relative populations are dictated by TFM incorporation. An exaggerated model depicting this concept is shown in Chart 1. The methionine positions are arbitrarily designated as 1, 2, and 3. An assumption is made that the TFM (large solid circle) at position 3 gives rise to the double resonance C/D. In situation A, position 2 is occupied by methionine (small open circle) and a particular local environment exists around the TFM at position 3, giving rise to resonance C. Incorporation of the more sterically demanding TFM at position 2 results in an altered protein conformation (situation B) which places the TFM at position 3 in a new environment, hence giving rise to resonance D. Our integration results appear to support such a scenario. In low-level TFM-LaL, position 2 should statistically be occupied with TFM 31% of the time when position 3 is TFM, and there would be a 2:1 ratio of resonance C to D as is observed. Conversely, high-level TFM would result in a 70% likelihood of TFM occupying position 2 when position 3 is TFM and there would exist a

2:1 ratio of D to C, and this is also observed. Since resonance C prevails under lower incorporation levels, it may be more indicative of the unperturbed structure. The model assumes that the two situations are not dependent on the occupancy of position 1, which could be Met or TFM (small solid circle). The chemical shift difference between C and D is very small (0.12 ppm) and could reflect very minor differences in the overall protein structures in the two situations. However, the chemical shift of fluorine is extremely sensitive to the local environment and even slight differences between the protein structures can generate sufficiently distinct microenvironments.

Novel fluorinated amino acid incorporation into peptides and proteins can be achieved by several methods both chemically and biosynthetically. Chemical approaches can include synthesis of polypeptides containing the analogue (Koul et al., 1979), by the use of selectively acylated suppressor tRNA's for *in vitro* protein expression (Noren et al., 1989), and by direct labeling of amino acid side chains with specific organic fluorinating reagents. The trifluoromethylmercury moiety has been selectively introduced into cysteine residues of actin and myosin using (trifluoromethyl)-mercuric bromide and ^{19}F NMR data on the chemically introduced protein label has been presented (Barden et al., 1989). However with the availability of TFM and having prepared an effective system for expression of LaL directed by the methionine auxotroph, we were able to establish biosynthetic conditions to control the overall substitution of the methionines in LaL with TFM and thereby making available a new probe to study protein structure and function. We have prepared TFM−LaL exhibiting wild type activity at high and low incorporation levels. Increased protein yields are obtained under the low level labeling conditions. In the application of L-TFM as a ^{19}F NMR probe for protein structure, it may be advantageous however to explore the effect of differential levels of incorporation of TFM into the particular protein of interest. The differences observed in the ^{19}F NMR spectra between high- and low-level TFM−LaL suggest that the structure of LaL is sensitive to the extent of TFM incorporation. Consequently, lower levels of incorporation may be more suitable to the study of less perturbed protein structures.

We have presented experimental evidence that labeling of LaL with TFM can be used to study its interactions with chitooligosaccharides by ^{19}F NMR. As exemplified by our current report, the utilization of TFM should be extremely useful in the study of protein−ligand and possibly protein−protein interactions by ^{19}F NMR. In proteins in which a specific function has been ascribed to particular methionine residues, the application of this approach may complement and offer additional insight on the roles and importance of this amino acid in protein structure and function.

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