

## Chapter 3

### Incorporation of Trifluoroisoleucine into Proteins *In Vivo*

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#### Abstract

Two fluorinated derivatives of isoleucine: D,L-2-amino-3-trifluoromethyl pentanoic acid (3TFI, **2**) and D,L-2-amino-5,5,5-trifluoro-3-methyl pentanoic acid (5TFI, **3**) were prepared. 5TFI was incorporated into a model target protein, murine dihydrofolate reductase (mDHFR), in an isoleucine auxotrophic *Escherichia coli* host strain suspended in 5TFI-supplemented minimal medium depleted of isoleucine. Incorporation of 5TFI was confirmed by tryptic peptide analysis and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of the protein product. Amino acid analysis showed that more than 93% of the encoded isoleucine residues were replaced by 5TFI. Measurement of the rate of activation of 5TFI by the *E. coli* isoleucyl-tRNA synthetase (IleRS) yielded a specificity constant ( $k_{cat}/K_m$ ) 134-fold lower than that for isoleucine. 5TFI was successfully introduced into the cytokine murine interleukin-2 (mIL-2) at the encoded isoleucine positions. The concentration of fluorinated protein that elicits 50% of the maximal proliferative response is 3.87 ng/ml, about 30% higher than that of wild-type mIL-2 ( $EC_{50}=2.70$  ng/ml). The maximal responses are equivalent for the fluorinated and wild-type cytokines, indicating that fluorinated proteins can fold into stable and functional structures. 3TFI yielded no evidence for *in vivo* incorporation into recombinant proteins, and no evidence for activation by IleRS *in vitro*.

## 1. Introduction

Protein engineering provides powerful tools for modification of natural proteins and for *de novo* design of artificial proteins (1, 2). Among the most recently developed protein engineering tools are methods for incorporation of non-canonical amino acids (2). Novel amino acids can be introduced by chemical methods such as solid-phase peptide synthesis and native chemical ligation (3). Expressed protein ligation and *in vitro* translation methods allow site-specific incorporation of non-natural amino acids (4-6). Most recently there has emerged an apparently general strategy for site-specific incorporation of non-canonical amino acids into proteins *in vivo* (7-9).

We have previously exploited the ability of auxotrophic *Escherichia coli* host strains to introduce amino acid analogs into proteins in multisite fashion. Functional groups distinct from those of the natural amino acids, including alkenes, alkynes, aryl halides, azides, ketones, pyridyls, and others have been incorporated into proteins *in vivo* with good efficiency (10-17). Multisite replacement is complementary to the site-specific method of analog insertion, and is of primary interest in situations where one wishes to change the overall physical behavior of the engineered protein.

Fluorinated amino acids are of special interest (18). Fluorine is not a sterically demanding substituent (van der Waals radii  $r_{(F)}=1.35 \text{ \AA}$ ,  $r_{(H)}=1.20 \text{ \AA}$ ) and although the C-F bond is  $0.4 \text{ \AA}$  longer than the C-H bond, introduction of fluorine generally causes minimal structural perturbation (19). Fluorocarbons yield higher contact angles with water compared to their hydrocarbon forms, indicating their elevated hydrophobicity (20). Fluorination is commonly employed in drug delivery to generate stable liposomes (21) and in drug design to increase drug activity (22), both of which result at least in part

from the enhanced hydrophobic character of fluorinated substituents. In light of fact that hydrophobic forces play important roles in protein folding and protein-protein recognition, it was suggested in a recent review that fluorinated amino acids could be utilized in protein engineering to prepare peptides and proteins with enhanced structural stability (23). We and Kumar and coworkers have recently demonstrated that substitution of leucine residues by 5,5,5-trifluoroleucine at the *d*-positions of the leucine zipper peptide GCN4-p1 increases the thermal stability of the coiled-coil structure (24-27). Furthermore we have been able to show that the fluorinated peptides maintain affinity and specificity in binding to their target DNA sequences (25). To date, it has been shown that mono-fluorinated analogs of phenylalanine (28), tryptophan and tyrosine (29), as well as trifluoroleucine (30), trifluoromethionine (31) and hexafluoroleucine (32) are translationally active. Each of these amino acids can be readily incorporated into target proteins in appropriate auxotrophic *E. coli* strains, though incorporation of hexafluoroleucine requires over-expression of the leucyl-tRNA synthetase of the host (32). In this work, we prepared two fluorinated analogs of isoleucine (**1**): D,L-2-amino-3-trifluoromethyl pentanoic acid (**2**, 3TFI) and D,L-2-amino-5,5,5-trifluoro-3-methyl pentanoic acid (**3**, 5TFI). We find that **3**, but not **2**, serves as an excellent surrogate for isoleucine in supporting protein synthesis in *E. coli*. Incorporation of **3** into the five isoleucine sites of murine interleukin-2 (mIL-2) yields a fluorinated variant characterized by activity nearly identical to that of the authentic cytokine.

## **2. Materials and Methods**

## 2.1. Synthesis of Amino Acid Analogs

Glassware was dried at 150 °C and cooled under nitrogen prior to use. 1,1,1-trifluoro-butan-2-one (**4**) was purchased from FluoroChem Inc. Unless otherwise specified, all reagents were used as received. Hydrogenation was performed with a Parr high-pressure hydrogenation apparatus (Westinghouse Electric Corp.). <sup>1</sup>H NMR and <sup>19</sup>F NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer.

D,L-2-Amino-5,5,5-trifluoro-3-methyl pentanoic acid (**3**) was prepared according to Muller *et al.* (33). This section provides detailed protocols for preparation of **2** (Scheme 1) and NMR data for intermediates prepared en route to **3** (Scheme 2)

*E,Z-3-Trifluoromethylpent-2-enoic acid ethyl ester.* Carboethoxymethylenetriphenylphosphorane (27.62 g, 80.5 mmol), 1,1,1-trifluoro-butan-2-one (**4**) (10 g, 79.4 mmol) and 70 ml ether were placed in a dry, 250 ml three necked flask. Reaction occurred immediately with formation of a thick paste. An additional 40 ml of ether was added and the mixture was refluxed for 24 hr at 50-60 °C with mechanical stirring. The reaction mixture was filtered and ether was removed *in vacuo*. The light yellow filtrate was subjected to vacuum distillation to afford 10 g (51 mmol, 63%) of a mixture of the E and Z forms of 3-trifluoromethyl-pent-2-enoic acid ethyl ester (94:6 by NMR). NMR assignments for the E and Z isomers were made according to previous reports on the synthesis of 4,4,4-trifluoro-3-methyl-but-2-enoic acid ethyl ester (41,42). <sup>1</sup>H NMR (CDCl<sub>3</sub>): (E) δ 1.06 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.23 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH), 2.67 (q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH), 4.2 (q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-O), 6.23 (s, 1H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH); (Z) δ 1.06 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.23 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH), 2.32

(q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH), 4.2 (q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-O), 6.05 (s, 1H, CH<sub>3</sub>-CH<sub>2</sub>-C(CF<sub>3</sub>)=CH).

*3-Trifluoromethylpentanoic acid ethyl ester (34)*. 3-Trifluoromethylpent-2-enoic acid ethyl ester (50 g, 255 mmol) was dissolved in 100 ml ethanol. PtO<sub>2</sub> (1.5 g) was added and the mixture was hydrogenated at 10 psi at room temperature for 12 hr. The catalyst was removed by filtration, 1 g of new PtO<sub>2</sub> was added and hydrogenation was continued for another 10 hr. The catalyst was filtered out and ethanol was removed *in vacuo*. Vacuum distillation yielded 35 g (176 mmol, 70%) of 3-trifluoromethylpentanoic acid ethyl ester. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.02 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 1.28 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.4-1.6 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 1.7-1.8 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 2.35-2.45 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 2.52-2.72 (m, 2H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 4.19 (q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-O).

*3-Trifluoromethylpentanoic acid (5) (35)*. 3-Trifluoromethylpentanoic acid ethyl ester (5 g, 22 mmol) was added to 50 ml of 1N of aqueous NaOH solution and the mixture was refluxed for 24 hr and then extracted with 50 ml of ether. The solution was acidified with HCl and extracted three times with 200 ml portion of ether. The combined ether layers were dried and ether was removed *in vacuo*. Vacuum distillation afforded pure **5** (2.55 g, 15 mmol, 70%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.02 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 1.54 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 1.76 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 2.45 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 2.65 (m, 2H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH<sub>2</sub>), 11.54 (s, 1H, COOH).

*Ethyl 2-bromo-3-trifluoromethylpentanoate*. To 5 g (29.4 mmol) of 3-trifluoromethylpentanoic acid at 30 °C, 4.2 g (35.3 mmol) of thionyl chloride was added

dropwise. The mixture was stirred at 60-80 °C until gas evolution essentially ceased. At 80 °C, 5.64 g (35.3 mmol) of Br<sub>2</sub> was added dropwise at a rate that matched the rate of consumption. Stirring was continued for several hours until the evolution of HBr stopped. The reaction mixture was cooled to room temperature and 60 ml of absolute ethanol was added slowly to the crude acid chloride. After standing overnight, the mixture was washed with water, and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>. Yield 6.5 g (23 mmol, 80%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.93 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(Br)), 1.25 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.48 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(Br)), 1.72 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(Br)), 2.35 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(Br)), 2.62 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(Br)), 4.18 (q, 2H, CH<sub>3</sub>-CH<sub>2</sub>-O).

*Ethyl 2-azido-3-trifluoromethylpentanoate (6).* Ethyl 2-bromo-3-trifluoromethylpentanoate (5 g, 18 mmol), 25 g (77 mmol) of sodium azide, 10 ml of ethanol and enough water to dissolve the sodium azide were refluxed for 6 days. The reaction mixture was distilled (CAUTION! Organic azides should be distilled with special care), and the organic layer was used for the next step without further purification. Yield 2.58 g (10.8 mmol, 60%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.98 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(N<sub>3</sub>)), 1.25 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.48 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(N<sub>3</sub>)), 1.7 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(N<sub>3</sub>)), 2.36 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(N<sub>3</sub>)), 2.5-2.7 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(N<sub>3</sub>)), 4.16 (q, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O). IR (CDCl<sub>3</sub>) 2100 cm<sup>-1</sup> (strong).

*Ethyl 2-amino-3-trifluoromethyl-pentanoate hydrochloride.* Ethyl 2-azido-3-trifluoromethylpentanoate (4 g, 16.7 mmol) was dissolved in ethanol (8 ml). Pd/C (0.04 g, 10% Pd) was added as catalyst and the mixture was hydrogenated at low pressure (10

psi) over 12 hr. The catalyst was filtered out and HCl in ether (40 ml) was added, after which the solvent was removed *in vacuo* to afford crude product (1 g, 4.7 mmol, 28%). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.02 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 1.23 (t, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.6-1.85 (m, 2H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 2.97 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 4.25 (m, 2H, CH<sub>3</sub>-CH<sub>2</sub>-O), 4.35 (t, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)).

*D,L*-2-Amino-3-trifluoromethylpentanoic acid (**2**). Ethyl 2-amino-3-trifluoromethylpentanoate (200 mg, 0.94 mmol) was dissolved in 6 N HCl and the solution was refluxed for 1 day. The mixture was evaporated to dryness *in vacuo*, and ethanol (5 ml) was added and again evaporated to dryness *in vacuo*. The resulting solid was dissolved in ethanol, neutralized with pyridine, and refrigerated over two days. The precipitate was filtered and dried *in vacuo* to afford practically pure amino acid (130 mg, 0.7 mmol, 74%). <sup>1</sup>H NMR (D<sub>2</sub>O) δ 0.85 (t, 3H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 1.3-1.5 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 1.5-1.8 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 2.81 (m, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)), 3.81/3.92 (d, 1H, CH<sub>3</sub>-CH<sub>2</sub>-CH(CF<sub>3</sub>)-CH(NH<sub>2</sub>)). <sup>19</sup>F NMR (D<sub>2</sub>O, TFA as reference) δ 7.1 (d, allo-isoleucine form), δ 9.6 (d, isoleucine form). MS(EI) molecular ion calcd for C<sub>6</sub>H<sub>10</sub>F<sub>3</sub>NO<sub>2</sub> 185.14, found 185.1.

*3-Methyl-but-3-enitrile* (**8**) (**36**). Cyanoacetic acid (100 g, 1.23 mol), 71.3 g (1.23 mol) of acetone, and 8.0 g (0.1 mol) of ammonium acetate were dissolved in 200 ml of dry benzene and the solution was subjected to reflux in a Dean-Stark apparatus overnight. The Dean-Stark apparatus was replaced with a distillation head and the desired product (52 g, 55%) was collected between 110 and 115 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.7-2.0 (s, 3H, CH<sub>2</sub>=C(CH<sub>3</sub>)-CH<sub>2</sub>-CN), 3.0 (s, 2H, CH<sub>2</sub>=C(CH<sub>3</sub>)-CH<sub>2</sub>-CN), 4.94 (s, H, CH<sub>2</sub>=C(CH<sub>3</sub>)-CH<sub>2</sub>-CN), 5.06 (s, H, CH<sub>2</sub>=C(CH<sub>3</sub>)-CH<sub>2</sub>-CN).

*3-Methyl-5,5,5-trifluoropentanitrile (9) (33)*.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.02/1.19 (d, 3H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.0-2.3 (m, 3H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.4 (d, 2H,  $\text{CH}(\text{CH}_3)\text{-CH}_2\text{-CN}$ ).

*2-Bromo-3-methyl-5,5,5-trifluoropentanoic acid (33)*.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.21 (dd, 3H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.0-2.3 (m, 1H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.3-2.8 (m, 2H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 4.21/4.5 (d, 1H,  $\text{CH}(\text{CH}_3)\text{-CH}(\text{Br})$ ), 11.6 (s, 1H,  $\text{COOH}$ ).

*D,L-2-Amino-5,5,5-trifluoro-3-methyl pentanoic acid (3) (33)*.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ )  $\delta$  0.96 (dd, 3H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.10-2.25 (m, 1H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 2.25-2.5 (m, 2H,  $\text{CF}_3\text{-CH}_2\text{-CH}(\text{CH}_3)$ ), 3.65 (d, 1H,  $\text{CH}(\text{CH}_3)\text{-CH}(\text{NH}_2)$ ).  $^{19}\text{F}$  NMR ( $\text{D}_2\text{O}$ , TFA as reference)  $\delta$  11.54 (t, 3F,  $\text{CF}_3$ ). MS(EI) molecular ion calcd for  $\text{C}_6\text{H}_{10}\text{F}_3\text{NO}_2$  185.14, found 185.1.

## 2.2. Determination of Translational Activity

Buffers and media were prepared according to standard protocols. An isoleucine auxotrophic derivative of *E. coli* strain BL21(DE3), designated AI (*E. coli* B  $\text{F}^-$  *ompT* *hsdS*( $\text{r}_\text{B}^-$   $\text{m}_\text{B}^-$ ) *gal dcm*  $\lambda$ (DE3) *ilvD691*), constructed in our laboratory, was used as host strain(37). Host cells of *E. coli* strain BW 6159 containing the *ilvD691*:Tn10 mutation were first infected by transducing phage P1 and the transposon was transferred into the expression strain BL21(DE3) (Novagen). The resulting auxotroph was treated with chlorotetracycline and fusaric acid to afford the stable auxotroph AI. The repressor plasmid pLysS-IQ was constructed by Sharma, and carried the *lacI<sup>q</sup>* gene for *lac* repressor (12). The AI strain with the repressor plasmid pLysS-IQ was designated AI-IQ.



The expression system AI-IQ[pQE15] was obtained by transformation of pQE15 (Qiagen) into AI-IQ.

### **2.3. Protein Expression and Incorporation of Trifluoroisoleucine into mDHFR**

Small-scale (5 ml) cultures were used to investigate the *in vivo* translational activity of **2** and **3**. M9 minimal medium (50 ml) supplemented with 0.2 % glucose, 1 mg/L thiamine, 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 19 amino acids (at 20 mg/L), antibiotics (ampicillin 200 mg/L, chloramphenicol 35 mg/L) and isoleucine (at 20 mg/L) was inoculated with 1 ml overnight culture of AI-IQ[pQE15]. When the optical density at 600 nm reached 0.8-1.0, a medium shift was performed. Cells were sedimented by centrifugation for 15 min at 4000 g at 4 °C, the supernatant was removed and the cell pellets were washed twice with 0.9% NaCl. Cells were resuspended in supplemented M9 medium containing either: (a) 500 mg/L analog (**2** or **3**), (b) 20 mg/L Ile (**1**) (positive control), (c) no Ile or analogs (negative control). Protein expression was induced 10 min after the medium shift by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were cultured for 4 hour post-induction and protein expression was monitored by SDS polyacrylamide gel electrophoresis (PAGE, 12 %), using a normalized OD<sub>600</sub> of 0.2 per sample.

Larger scale expression of mDHFR was performed in 1 L cultures of AI-IQ[pQE15] grown in the supplemented M9 medium described above. Cells were sedimented and washed twice with 0.9 % NaCl and resuspended in supplemented M9 medium containing **3** at 500 mg/L. Gene expression was induced with 1 mM IPTG 10 min after the medium shift.

#### **2.4. Protein Purification**

The mouse dihydrofolate reductase (mDHFR) encoded in pQE15 carries a 6xHis tag sequence at the N-terminus. mDHFR was purified by nickel affinity chromatography with stepwise pH gradient elution under denaturing conditions according to the recommendations of the supplier (Qiagen). The eluted protein was dialyzed (Spectra/Por membrane 1, MWCO=6-8 kDa) against distilled water for 2 days and lyophilized. The purified protein was subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and amino acid analysis.

#### **2.5. Tryptic Peptide Analysis**

Purified protein (10  $\mu$ L) in elution buffer (8 M urea, 100 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM Tris, pH=4.5) was mixed with 90  $\mu$ l 75 mM  $\text{NH}_4\text{OAc}$  and 2  $\mu$ L of modified trypsin (Promega, 0.2  $\mu$ g/ $\mu$ L). The solution was incubated overnight at room temperature, and quenched by addition of trifluoroacetic acid (pH < 4.0). The tryptic digest was then subjected to sample clean-up by ZipTip<sub>C18</sub> to provide 2  $\mu$ l of purified sample solution. The MALDI matrix  $\alpha$ -cyano- $\beta$ -hydroxycinnamic acid, (10  $\mu$ l, 10 mg/ml in 50%  $\text{CH}_3\text{CN}$ ) was added to this solution, and 0.5  $\mu$ l of the resulting solution was spotted directly on the sample plate. Samples were analyzed on an Applied Biosystems Voyager mass spectrometer operating in linear mode.

#### **2.6. IleRS Cloning, Expression and Purification**

The isoleucyl-tRNA synthetase (IleRS) gene was cloned directly from *E. coli* genomic DNA with two flanking primers encoding the desired restriction sites *Bam*HI and *Sac*I (primer 1: 5'-CAA ACC GAA ATA CGG ATC CGA GAA TCT GAT G-3'; primer 2: 5'-CTG TTG AAC AGA TCG ATT GAG CTC ATC AGG CAA ACT TAC-3'). The resulting 3000 base-pair DNA fragment was gel-purified, digested with *Bam*HI and *Sac*I and ligated into the expression plasmid pQE30 (Qiagen) to yield pQE-ileS. The integrity of the cloned gene was confirmed by DNA sequencing. The cloned synthetase carried the N-terminal leader sequence MRGSHHHHHHGSENL. pQE-ileS was transformed into XL-1 (Stratagene) to yield the expression strain. Protein expression was induced at OD<sub>600</sub>=0.6 with 1 mM IPTG. After three hours, the cells were harvested. The enzyme was purified using Ni-NTA agarose resin under native conditions according to the manufacturer's instructions (Qiagen). Protein was stored in Buffer A (50 mM Tris-HCl, 1 mM DTT)/50% glycerol. Aliquots were flash frozen and stored at -80 °C. The concentration of the purified enzyme was determined by absorbance at 280 nm under denaturing conditions.

## 2.7. ATP-PP<sub>i</sub> Exchange Assay

Measurement of the rates of activation of isoleucine analogs by IleRS was carried out via an ATP-PP<sub>i</sub> exchange assay as described by Schimmel and coworkers (38). The assay buffer contained 30 mM HEPES, pH 7.4, 10 MgCl<sub>2</sub>, 1 mM DTT, 2 mM ATP and 2 mM [<sup>32</sup>P]-PP<sub>i</sub> (0.5 TBq/mol). The enzyme concentration was 100 nM. The amino acid concentration varied depending on the activity of enzyme toward the substrate (**1**: 10-500 μM; **2, 3**: 30-10,000 μM). Aliquots (20 μL) of reaction mixture were quenched into 500

$\mu$ L quench buffer (200 mM  $\text{PP}_i$ , 7% w/v  $\text{HClO}_4$  and 3% activated charcoal). The charcoal was washed twice with wash buffer (10 mM  $\text{PP}_i$ , 0.5%  $\text{HClO}_4$ ) and counted by liquid scintillation methods. Kinetic parameters were obtained by nonlinear regression analysis.

## **2.8. Incorporation of 5TFI into Murine Interleukin-2**

Murine interleukin-2 (mIL-2) was cloned into pQE31 (Qiagen) by PCR cloning through restriction sites *Bam*HI and *Sal*I. The cloned gene was confirmed by DNA sequencing. The resulting plasmid pQE-mIL2 was transformed into AI-IQ to yield expression strain AI-IQ[pQE-mIL2]. Expression was performed essentially as described above. Tryptic peptide digestion was employed to confirm the incorporation of **3** into mIL-2. Protein was first purified using Ni-NTA agarose resin under denaturing conditions according to the manufacturer's instructions (Qiagen). His-tag purified fractions were pooled and diluted in 8 M urea to a final protein concentration of less than 25 mg/L. The protein was refolded according to a literature procedure (39). The diluted protein solution was step-wise dialyzed against the following solutions: 1) 6 M urea, 2.5 mM DTT, 2.5 mM  $\beta$ -mercaptoethanol ( $\beta$ -ME), 10 mM Tris·HCl, pH=7.4; 2) 4 M urea, 5 mM  $\beta$ -ME, 10 mM Tris·HCl, pH=7.4; 3) 2 M urea, 5 mM  $\beta$ -ME, 10 mM Tris·HCl, pH=7.4; 4)  $\text{H}_2\text{O}$ , 5 mM  $\beta$ -ME; 5)  $\text{H}_2\text{O}$ , 1 mM DTT; 6) HEPES-buffered saline, 1 mM DTT; 7) 50 mM HEPES, pH=7.4, 1 mM DTT. The renatured protein was concentrated via ultrafiltration and stored at  $-80^\circ\text{C}$ . Protein concentration was measured using a Bio-Rad protein binding assay kit with bovine serum albumin (BSA) as a standard.

Biological activity of mIL-2 was determined by a proliferation assay using the IL-2 dependent H2-T cell line (ATCC) (40). The serial 10-fold dilutions of the renatured protein sample were incubated with H2-T cells in 96-well plates ( $1 \times 10^3$  cells perwell) overnight. Then cells were incubated with  $^3\text{H}$ -thymidine for 5 hours and harvested onto filter paper and analyzed on a Wallac (Gaithersburg, MD) 96-well counter.

### 3. Results

#### 3.1. Synthesis of Isoleucine Analogs

3TFI was prepared from 1,1,1-trifluoro-2-butanone (**4**) as shown in Scheme 1. Treatment of the fluoroketone with carboethoxymethylenetriphenylphosphorane gave a mixture of E and Z isomer of 3-trifluoromethyl-2-pentenoic acid ethyl ester (94:6) (41, 42), which was converted to the saturated ester by low pressure hydrogenation. Hydrolysis to the acid (**5**), followed treatment with thionyl chloride and bromine, and finally quenching with ethanol afforded ethyl 2-bromo-3-trifluoromethylpentanoate. Walborsky et al found that ammonolysis of ethyl 2-bromo-4,4,4-trifluorobutyrate gave 3-amino-4,4,4-trifluorobutyramide instead of the desired 2-amino isomer (43). By using azide ion to decrease the tendency for elimination and to favor direct displacement of bromide, Loncirni et al obtained ethyl 2-azido-3,3,3-trifluorobutyrate (44). We used a similar procedure to prepare ethyl 2-azido-3-trifluoromethylpentanoate (**6**), which after catalytic hydrogenation and hydrolysis provided 3TFI in an overall yield of 5% based on **4**. The resulting mixture of diastereomers of 3TFI was used in studies of incorporation into proteins *in vivo*.

5TFI was prepared according to Muller (45) as shown in Scheme 2. One pair of enantiomers, corresponding to 5,5,5-trifluoro-DL-isoleucine, was separated by fractional crystallization. We use the designation 5TFI to refer to the enantiomeric pair.

### **3.2. Incorporation of 5TFI into Protein *In Vivo***

We used *E. coli* strain AI-IQ[pQE15] to evaluate production of the test protein mDHFR in cultures supplemented with 3TFI and with 5TFI, respectively. The parent isoleucine auxotrophic strain AI was generated from *E. coli* strain BL21(DE3) by P1-mediated transduction using a donor strain (BW 6159), which had been transposed by Tn10 at the *ileD* gene region (37); *ileD* is essential for the endogenous synthesis of isoleucine and valine(46). The AI-IQ[pQE15] culture was grown to  $OD_{600} = 1.0$  in supplemented M9 minimal medium, then shifted to a medium containing either: a) the analog of interest, b) Ile (positive control) or c) no Ile or analog (negative control). Protein synthesis was induced by addition of IPTG. The results of expression of mDHFR are shown by SDS-PAGE analysis of whole cell lysates (Figure 3-1). No expression of mDHFR was detected in negative control cultures or in cultures containing 3TFI (500 mg/L). In contrast, synthesis of mDHFR is evident in cultures supplemented with Ile (20 mg/L) or with 5TFI (500 mg/L). We also explored a modified expression host (AI-IQ[pQE15-IRS]), in which the isoleucyl-tRNA synthetase was over-expressed. Amino acid activation assays on whole cell lysates indicated that the IleRS activity of the modified host was elevated approximately 6-fold as compared to the wild-type host. Nevertheless, qualitatively similar results were obtained from incorporation experiments on modified and wild-type strains (Figure 3-1).

After purification by nickel-affinity chromatography, the yield of protein (mDHFR-5TFI) expressed in 5TFI-supplemented medium was 20 mg/L, approximately 60% of that isolated from the positive control medium (Table 3-1). Amino acid analysis showed the extent of substitution of 5TFI for Ile to be 93%. Purified mDHFR was also subjected to MALDI-TOF mass spectrometry. The difference in molar mass between mDHFR-5TFI and mDHFR-wt is 755 Da (Table 3-1), consistent with the fact that there are 14 isoleucine residues in mDHFR and with the mass difference of 54 Da between 5TFI and Ile.

Incorporation of 5TFI was also confirmed by tryptic peptide analysis of purified mDHFR. Figure 3-2a shows a representative tryptic fragment of mDHFR-wt with a mass of 2674.15 Da. This fragment is assigned to residues 124-147 (Figure 3-2a) and contains 1 of the 14 isoleucine residues of mDHFR. For mDHFR-5TFI, the mass of this fragment is shifted to 2728.04 Da (Figure 3-2b). The additional mass (53.88 Dalton) corresponds to the mass difference between 5TFI and Ile. The original signal has disappeared, consistent with near quantitative substitution of Ile by 5TFI.

### **3.3. Effect of 5TFI on Cell Growth**

We also determined the effect of 5TFI on the growth of the *E. coli* host strain prior to induction. Isoleucine is required for growth of *E. coli* strain AI. In cultures supplemented with 0.2 mM isoleucine, the rate of cell growth is dependent on the concentration of 5TFI. At an analog concentration of 4 mM, cell growth is completely inhibited (Figure 3-3). The dose-dependent toxicity of 5TFI presumably arises from incorporation of the fluorinated amino acid into cellular proteins.

### 3.4. Amino Acid Activation *In Vitro*

The rates of ATP-dependent activation of isoleucine and isoleucine analogs **2** and **3** by *E. coli* isoleucyl-tRNA synthetase (IleRS) were examined by the ATP-PP<sub>i</sub> exchange assay. The kinetic parameters are reported in Table 3-2. The values of  $K_m$  and  $k_{cat}$  for Ile are in the range of values reported in the literature (47-50). Because 3TFI causes no exchange of PP<sub>i</sub> above background level, no kinetic parameters were obtained. Comparison of the specificity constants ( $k_{cat}/K_m$ ) obtained for isoleucine ( $0.44 \text{ s}^{-1}\mu\text{M}^{-1}$ ) and for 5TFI ( $3.28 \times 10^{-3} \text{ s}^{-1}\mu\text{M}^{-1}$ ) show that the fluorinated analog is activated roughly 100-fold less efficiently than the natural substrate.

### 3.5. 5TFI in Murine Interleukin-2

5TFI was efficiently incorporated into mIL-2, as shown by SDS-PAGE and MALDI analysis of tryptic peptide fragments (Figure 3-4). Amino acid analysis indicated that 85% of isoleucine was replaced by **3**. Refolded mIL-2 samples were analyzed for activity by measuring the rate of [<sup>3</sup>H]-thymidine incorporation in the mIL-2 dependent cell line H2-T stimulated by varying concentrations of the wild-type and fluorinated proteins. Dose-response curves were similar for both forms of the protein, as shown in Figure 3-5. As shown in Table 3-3, the concentration of fluorinated protein that elicits 50% of the maximal proliferative response is 3.87 ng/ml, about 30% higher than that of wild-type mIL-2 ( $EC_{50}=2.70 \text{ ng/ml}$ ). The maximal responses are equivalent for the fluorinated and wild-type cytokines.



#### 4. Discussion

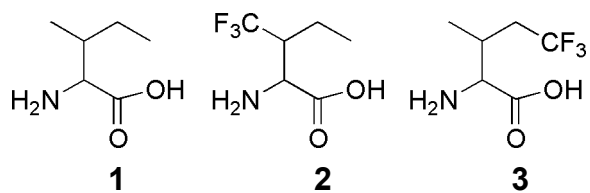
Prior to the start of this work, it was known that isoleucine analogs 2-amino-3-methylthiobutyric acid, 2-amino-3-methylaminobutyric acid (51), O-methylthreonine (52) and  $\beta$ -methylnorleucine (53) inhibit the growth of *E. coli* at millimolar concentrations, suggesting that these analogs might serve as substrates for the isoleucyl-tRNA synthetase and find their way into cellular proteins. Our results show clearly that 5TFI serves as an effective isoleucine surrogate in the translation step of protein biosynthesis in *E. coli*. 3TFI behaves in a strikingly different manner: no evidence for incorporation has been found. The fact that we did not observe incorporation of 3TFI prompted us initially to engineer an *E. coli* host strain with enhanced IleRS activity, but we found that 3TFI does not support measurable protein synthesis even with over-expression of IleRS.

The results of *in vitro* activation assays are consistent with the *in vivo* results. 5TFI shows modest activity ( $k_{cat}/K_m$  134-fold less than that of isoleucine), whereas only background levels of pyrophosphate exchange were observed in experiments with 3TFI. The origins of the difference in activity are under investigation.

The high fidelity of IleRS is due in part to the editing activity of the synthetase. A double-sieve mechanism has been proposed by Fersht and coworkers (54) and verified in structural studies (55). According to this model, IleRS has two sites for amino acid discrimination: the synthetic site serves as a coarse sieve and precludes amino acids larger than Ile from binding; the editing site serves as a fine sieve that removes misactivated amino acids smaller than Ile. Our *in vitro* results suggest that the synthetic

site binds 5TFI, but not 3TFI. Results from *in vivo* incorporation studies confirm that 5TFI is not edited, consistent with the enhanced steric bulk of the fluorinated side chain.

The extent to which globular proteins can tolerate incorporation of non-canonical amino acids without loss of function remains largely unexplored. Interleukin-2 (IL-2) is one of the major cytokines mediating the immune system, with the responsibility of T cell clonal expansion after antigen recognition (56). The crystal structure of human IL-2, a four-helix bundle protein, is shown in Figure 3-6 (57). The sites of interaction with the IL-2 receptor are shown in blue (58). All 5 isoleucine residues in mIL-2 are conserved in hIL-2 and are located in the helical core structure (Figure 3-6). Amino acid analysis indicated 85% substitution of Ile by 5TFI in the sample of mIL-2 prepared here; thus the fraction of wild-type cytokine in the fluorinated sample is negligible. The proliferative response elicited by fluorinated mIL-2 indicates that the fluorinated protein must fold into an authentic, native structure. The generality of this result remains to be determined; however, these observations coupled with our previous reports (25, 30, 32) suggest that at least some protein domains will tolerate side-chain fluorination without loss of function.



**Table 3-1. Protein Yield and Extent of Isoleucine Replacement in mDHFR**

Protein	Yield (mg/L)	Mass (Da)	5TFI replacement (%)	
			AAA	MALDI
mDHFR-WT	30	24063		
mDHFR-5TFI	20	24818	93.2	99.8

**Table 3-2. Kinetic Parameters for Activation of 1 and 3 by *E. coli* IleRS.**

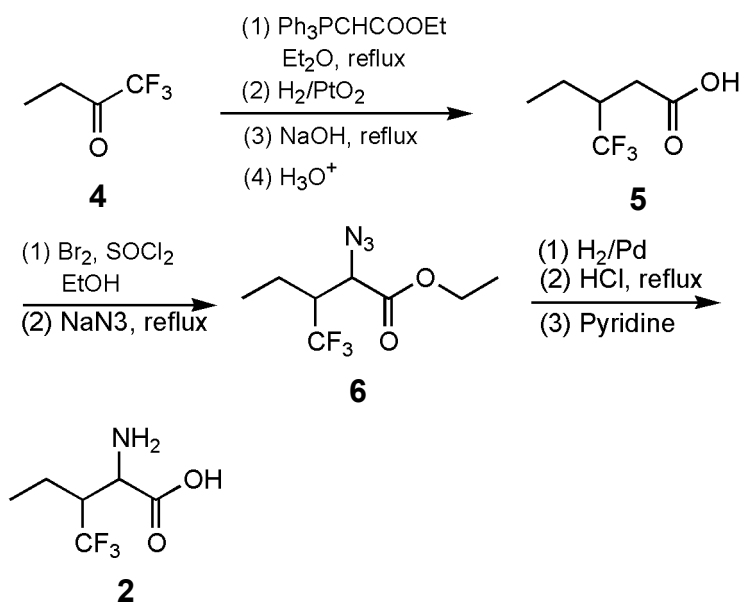
Amino Acid	$K_m$ ( $\mu\text{M}$ )	$k_{cat}$ (1/s)	$k_{cat}/K_m$ (rel)
<b>1</b>	6.82	3.0	1
<b>3</b>	30.52	0.1	1/134

**Table 3-3. Proliferative Response of H2-T Cells**

Protein	EC <sub>50</sub> <sup>a</sup> (ng/ml)
mIL2-wt	2.70±0.31
mIL2-5TFI	3.87±0.22

<sup>a</sup> Concentration of protein eliciting 50% of maximal response as measured by proliferation assay. Results from triplicate determinations.

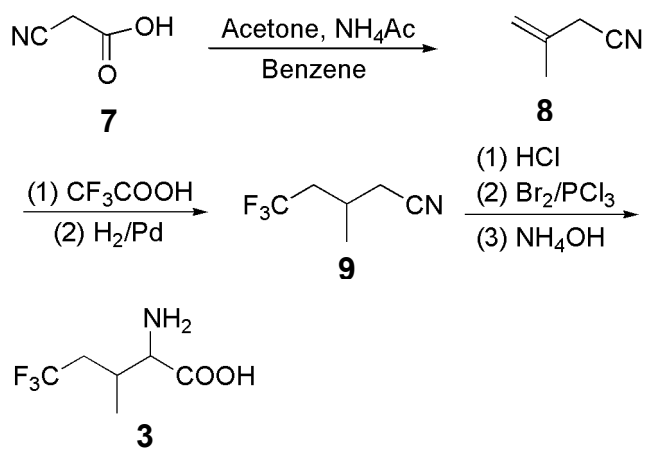
## Scheme 1







## Scheme 2



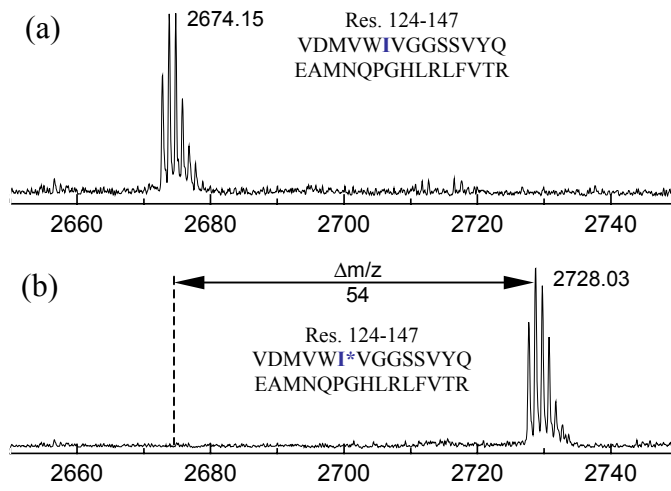
**Figure 3-1**

SDS-PAGE analysis of mDHFR synthesized by AF-IQ[pQE15] or AF-IQ[pQE-IleRS]. Expression conditions are noted above each lane. The lane in the middle is the molecular weight marker. pQE-IleRS is the plasmid derived from pQE15 (Qiagen), with insertion of the IleRS over-expression cassette at the *NheI* site.



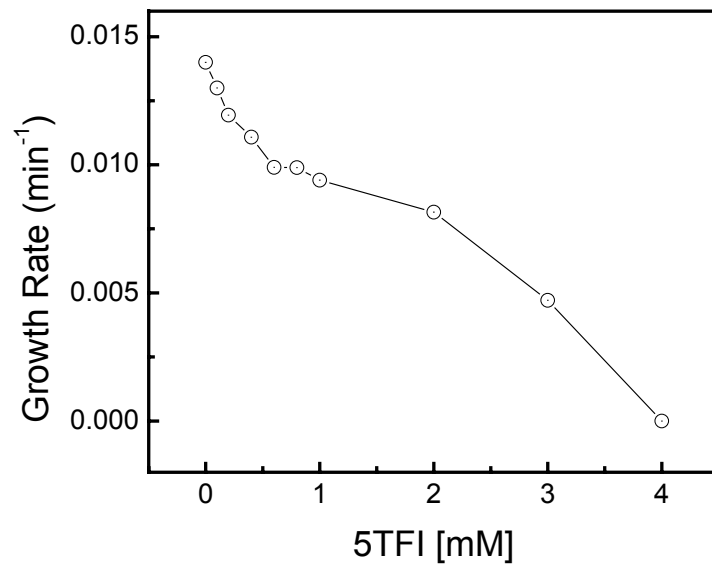
**Figure 3-2**

MALDI analysis of mDHFR following trypsin digestion. A fragment of sequence VDMVWIVGGSSVYQEAMNQPGLRRLFVTR, yields the spectra shown. (a) fragment digested from mDHFR-WT; (b) fragment digested from mDHFR-5TFI.



**Figure 3-3**

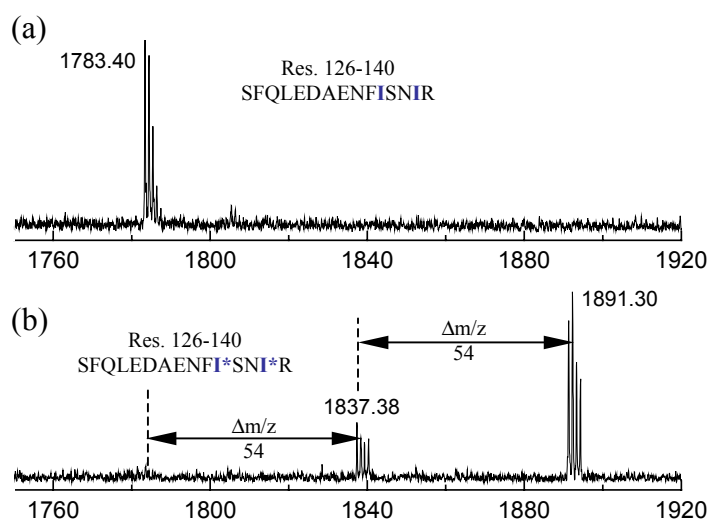
Growth rate of *E. coli* strain AF-IQ[pQE15] as a function of the concentration of 5TFI in the culture medium.



**Figure 3-4**

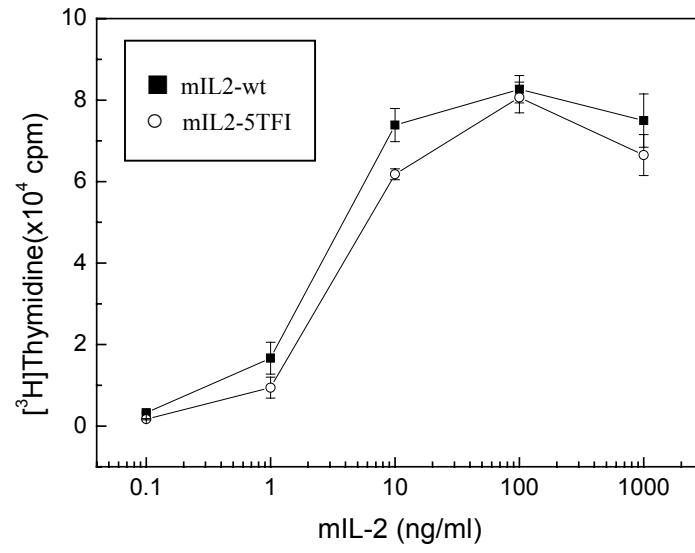
MALDI analysis of mIL-2 following trypsin digestion. A fragment of sequence SFQLEDAENFISNIR yields the spectra shown. (a) Fragment digested from wild-type mIL-2; (b) fragment digested from mIL-2 containing 5TFI.





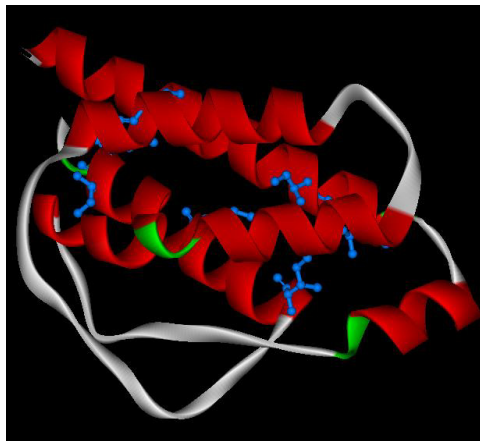
**Figure 3-5**

Proliferative response of IL-2 dependent H2-T cells to recombinant mIL-2 proteins: wild-type mIL-2 (■); mIL-2 containing 5TFI (○). The results are averages from triplicate experiments.



**Figure 3-6**

Crystal structure of hIL-2 (PDB: 1M47). The identified sites of interaction of hIL-2 with its receptor are shown in blue. The five conserved isoleucine residues are represented by ball-and-stick models.



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