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Recognition of *Escherichia coli* Valine Transfer RNA by Its Cognate Synthetase: A Fluorine-19 NMR Study†

Wen-Chy Chu and Jack Horowitz*
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011
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ABSTRACT: Interactions of 5-fluorouracil-substituted *Escherichia coli* $tRNA^{Val}$ with its cognate synthetase have been investigated by fluorine-19 nuclear magnetic resonance. Valyl-tRNA synthetase (VRS) (EC 6.1.1.9), purified to homogeneity from an overproducing strain of *E. coli*, differs somewhat from VRS previously isolated from *E. coli* K12. Its amino acid composition and N-terminal sequence agree well with results derived from the sequence of the VRS gene [Heck, J. D., & Hatfield, G. W. (1988) *J. Biol. Chem. 263*, 868-877]. Apparent K_M and V_{max} values of the purified VRS are the same for both normal and 5-fluorouracil (FUra)-substituted $tRNA^{Val}$. Binding of VRS to (FUra)$tRNA^{Val}$ induces structural perturbations that are reflected in selective changes in the ^{19}F NMR spectrum of the tRNA. Addition of increasing amounts of VRS results in a gradual loss of intensity at resonances corresponding to FU34, FU7, and FU67, with FU34, at the wobble position of the anticodon, being affected most. At higher VRS/tRNA ratios, a broadening and shifting of FU12 and of FU4 and/or FU8 occur. These results indicate that VRS interacts with $tRNA^{Val}$ along the entire inside of the L-shape molecule, from the acceptor stem to the anticodon. Valyl-tRNA synthetase also causes a splitting of resonances FU55 and FU64 in the T-loop and stem of $tRNA^{Val}$, suggesting conformational changes in this part of the molecule. No ^{19}F NMR evidence was found for formation of the Michael adduct between VRS and FU8 of 5-fluorouracil-substituted $tRNA^{Val}$ that has been proposed as a common intermediate in the aminoacylation reaction.

Aminoacyl-tRNA synthetases catalyze the first step in protein biosynthesis, i.e., attachment of a specific amino acid to the 3′ end of its cognate tRNA. Because the fidelity of translation depends in large part on the specificity of this reaction, numerous experimental approaches have been employed to investigate the interaction between tRNA and aminoacyl-tRNA synthetases, including aminoacylation studies of chemically modified, mutant, or dissected tRNAs; protection

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* Address correspondence to this author.

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of tRNA by synthetase against chemical modification and nuclease digestion; and direct mapping of enzyme–tRNA contact points by photochemical cross-linking of tRNA and synthetase [reviewed by Schimmel and Söll (1979) and Schimmel (1987)]. Results indicate that the detailed mechanism of tRNA recognition is likely to vary for different cognate systems (Normanly & Abelson, 1989; Schimmel, 1989), which is not surprising, given the diversity in subunit structure and size of the syntheses (Schimmel & Söll, 1979).

More direct structural methods, including X-ray crystallography, have also been used to investigate the synthetase–tRNA complex (Lorber et al., 1983; Podjarny et al., 1987; Perona et al., 1988; Rould et al., 1989). The recent results of Steitz and co-workers on the crystal structure of *Escherichia coli* glutaminyl-tRNA synthetase complexed with $tRNA^{Gln}$ and ATP (Perona et al., 1988; Rould et al., 1989) provide important detailed insights into the conformational changes occurring as a result of synthetase–tRNA interaction in this cognate system. Nuclear magnetic resonance (NMR)[1] provides another approach to the direct investigation of nucleic acid structure. Although ^1H NMR spectroscopy has been widely used to probe the dynamic structure of tRNA (Reid, 1981), few ^1H NMR studies of tRNA–synthetase interactions have been reported (Schulman et al., 1974). This is due in part to the small chemical shift range of the ^1H nucleus, with corresponding difficulties of resolution and peak assignment, and also partly to background interference from the protein.

Some of these difficulties can be overcome by use of other magnetic nuclei. In recent reports, we have described the successful application of ^{19}F NMR as a probe of tRNA structure, using tRNA molecules labeled with fluorine by substitution with 5-fluorouracil (FUra) (Hills et al., 1983; Gollnick et al., 1986, 1987; Hardin et al., 1986, 1987, 1988; Chu & Horowitz, 1989). The high sensitivity to detection (83.3% of ^1H), 100% natural abundance, large chemical shift range, and extreme sensitivity of ^{19}F chemical shifts to the environment of the fluorine nucleus (Sykes & Hull, 1978; Gerig, 1978; Sykes & Weiner, 1980) make ^{19}F NMR a promising tool for the study of fluorinated nucleic acids. It is especially well suited to probe protein–nucleic acid complexes because spectral changes can be observed without interference from background protein signals.

E. coli $tRNA^{Val}$ is the most thoroughly characterized fluorine-substituted tRNA (Gollnick et al., 1986, 1987; Hardin et al., 1986, 1987, 1988; Chu & Horowitz, 1989). It is ideal for ^{19}F NMR investigations because it retains its physical properties (Horowitz et al., 1974) and remains fully active in protein synthesis in vitro (Ofengand et al., 1974), despite high levels of FUra incorporation.

The ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ shows a resolved resonance for each incorporated FUra residue (Hardin et al., 1986, 1988; Chu & Horowitz, 1989). We have recently completed assignment of the spectrum (Chu et al., submitted for publication), and in the experiments reported here, we have used ^{19}F NMR spectroscopy to explore the interactions of *E. coli* valyl-tRNA synthetase with fluorine-labeled *E. coli* $tRNA^{Val}$. By monitoring the perturbations in the ^{19}F spectrum on the binding of VRS, we can infer sites of enzyme interaction.

[1] Abbreviations: NMR, nuclear magnetic resonance; FUra, 5-fluorouracil; Furd, 5-fluorouridine; (FUra)$tRNA^{Val}$, 5-fluorouracil-substituted *E. coli* $tRNA^{Val}$; VRS, *E. coli* valyl-tRNA synthetase; SIS, solvent isotope shift; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Experimental Procedures

Materials

Sepharose 4B, HEPES, phenylmethanesulfonyl fluoride (PMSF), and protein standards for SDS gel electrophoresis were all obtained from Sigma. Affi-Gel blue gel, hydroxylapatite, and Bradford protein assay reagent were purchased from Bio-Rad Laboratories; ammonium sulfate (Ultrapure) from Schwarz/Mann; unfractionated *E. coli* tRNA from Plenum Scientific Research Inc.; native *E. coli* $tRNA^{Val}$ from Subriden RNA; radioactive valine from New England Nuclear or ICN; dithiothreitol (DTT) from CalBiochem; and materials used to prepare media for cell growth from Difco or Fisher Chemical Co. Other reagents were of analytical grade or higher. 5-Fluorouracil-substituted *E. coli* $tRNA^{Val}$ was synthesized by in vitro transcription with T7 RNA polymerase from a recombinant phagemid containing the tRNA gene joined directly to an upstream T7 promoter (Chu & Horowitz, 1989).

Methods

Purification of Valyl-tRNA Synthetase (VRS). Valyl-tRNA synthetase was prepared by modification of the method of Eldred and Schimmel (1972), from *E. coli* GRB238/pHOV1 grown to late log phase at 40 °C in the medium described by Skogman and Nilsson (1984). All steps were performed at 4 °C. Cells, suspended in 100 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 1 mM DTT, and 0.2 mM PMSF, were disrupted by passage through a French pressure cell (American Instrument). The crude extract was autolyzed by incubation at 37 °C for 3 h to destroy nucleic acids and then fractionated by addition of solid ammonium sulfate. Protein precipitated between 45% and 60% saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 2 M ammonium sulfate, and VRS was further purified by chromatography on a Sepharose 4B column developed with a linear reverse gradient of ammonium sulfate from 2 to 0 M. Fractions containing enzyme activity were pooled and concentrated by ammonium sulfate precipitation (80% saturation), and the protein precipitate, dissolved in 20 mM potassium phosphate, pH 7.0, 0.5 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM PMSF, and 25% glycerol, was loaded onto a hydroxylapatite column. The peak of VRS activity, eluted with a linear gradient of potassium phosphate from 35 to 150 mM, was pooled and concentrated by using a PM 10 Diaflo membrane in an Amicon ultrafiltration cell. In the final purification step, the enzyme was applied to an Affi-Gel blue gel column equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 15 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, and 25% glycerol, and eluted with a linear gradient of NaCl from 0.05 to 1.5 M. Purified VRS was concentrated by pressure filtration as described above and stored in 50% glycerol at −70 °C at a concentration of 3 mg/mL. Enzyme yields were as high as 150 mg from 216 g of cells, with a 50% recovery of activity.

Protein concentration was determined by the dye binding assay of Bradford (1976), using bovine serum albumin as standard. The purification procedure was monitored by electrophoresis on 10% SDS–PAGE, as described by Laemmli (1970). Gels were stained with 1% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid.

Amino Acid Analysis and Amino-Terminal Sequence Determination of VRS. Purified VRS, dialyzed against H_2O,

was hydrolyzed with 6 N HCl at 165 °C for 1 h. Amino acids were derivatized with PITC on an Applied Biosystem 420A derivatizer and were separated by a reverse-phase column on an Applied Biosystem 130A system. PTH-amino acids were detected at 254 nm.

The amino-terminal sequence of the protein was determined by automatic Edman degradation with an Applied Biosystem 470A protein sequencer and a 120A PTH analyzer by Dr. L. Tabatabai at the National Animal Disease Center, Ames, IA.

Aminoacylation Reactions. Enzyme activity during the purification of VRS was determined by aminoacylation assay in a 100-μL reaction mixture containing 100 mM HEPES, pH 7.5, 10 mM KCl, 10 mM ATP, 1 mM DTT, 20 mM $MgCl_2$, 5 nmol of unfractionated *E. coli* tRNA, and 3.33 nmol of [^{14}C]valine (90 mCi/mmol) or 0.5 nmol of [^{3}H]valine (2 mCi/μmol). Reactions were incubated for either 10 min at 30 °C (^{14}C-amino acid) or 6 min at 37 °C (^{3}H-amino acid). Two milliliters of cold 5% trichloroacetic acid was added to stop the reaction. After 10 min on ice, the precipitates were collected on Millipore filters, dried, and counted in a Beckman LS-100C scintillation counter in a toluene-based scintillation cocktail containing 0.5% PPO and 0.01% POPOP. One unit of enzyme is defined as the amount of enzyme aminoacylating 1 μmol of [^{14}C]valine to tRNA in 10 min at 30 °C.

The catalytic activity of purified VRS was determined by charging transfer RNA at 25 °C in a 50-μL reaction mixture containing 100 mM HEPES, pH 7.5, 10 mM KCl, 15 mM $MgCl_2$, 7 mM ATP, 1 mM DTT, 150 μM [^{14}C]valine (133 mCi/mmol), 1 ng of purified enzyme, and varying amounts of tRNA, 0.3–2.7 μM. The mixture was preincubated at 25 °C for 5 min before enzyme was added. Reactions were stopped after 5 min by addition of cold 5% trichloroacetic acid. The precipitates were collected and counted, as described above. Preliminary experiments showed that the rate of aminoacylation was constant for at least 6 min.

^{19}F NMR Spectroscopy. Transfer RNA samples (3–7 mg) were dissolved in NMR buffer containing 55.55 mM sodium cacodylate, pH 6.0, 16.66 mM $MgCl_2$, 11.11 mM NaCl, and 1.11 mM EDTA and dialyzed against two changes of 250 mL of the same buffer in a flow-dialysis microcell (BRL Model 1200 MA). Sample volume was then adjusted to 300 μL, and 33 μL of D_2O was added to provide an internal lock signal. In vitro transcribed tRNA was renatured by heating at 55 °C for 20 min and slowly cooling to room temperature before transfer to a Wilmad 529A-10 spherical NMR microcell. This was suspended in a 10-mm NMR tube containing 1 mM 5-fluorouracil in sample buffer to serve as an external standard. To determine solvent isotope shifts, samples were transferred from 10% D_2O to 100% D_2O by lyophilizing twice and dissolving in 99.96% D_2O (Sigma).

To record spectra of the tRNA–VRS complex, we added VRS in NMR buffer (10% D_2O) to the tRNA sample to give the desired VRS to tRNA ratio. Volume was reduced to 330 μL by ultrafiltration in a Centricon-10 microconcentrator (Amicon), and the solution was transferred to an NMR tube as described above.

^{19}F NMR spectra were collected at 282 MHz on a Bruker WM-300 pulsed FT NMR spectrometer at 30 °C. Spectra were accumulated by using 8K data points, no relaxation delay, and a pulse width sufficient to optimize the Ernst condition (Shaw, 1976). Chemical shifts are reported as ppm from free 5-fluorouracil; downfield shifts are indicated as positive.

Results

Purification and Characterization of E. coli Valyl-tRNA Synthetase. To obtain the large quantities of protein required for structural studies, VRS was isolated from an overexpressing strain of *E. coli*, GRB 238/pHOV1 (Skogman & Nilsson, 1984). Although VRS had previously been isolated from *E. coli* K12 (Yaniv & Gros, 1969), we briefly describe the enzyme isolated from *E. coli* GRB 238/pHOV1 because its properties differ in several respects from those reported for the *E. coli* K12 synthetase.

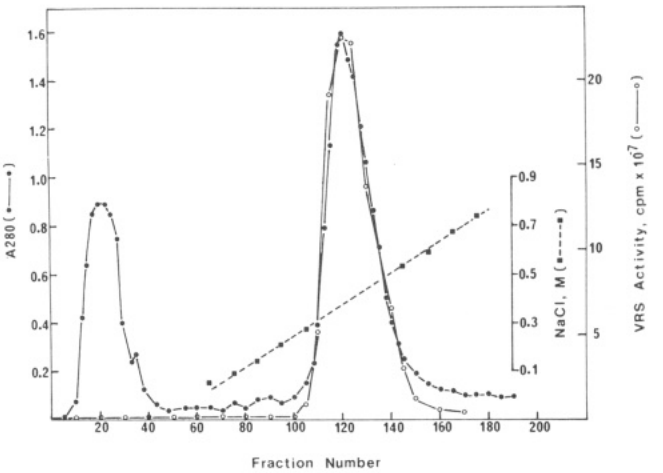

FIGURE 1: Affi-Gel blue gel chromatography of *E. coli* valyl-tRNA synthetase. Valyl-tRNA synthetase (9.2 mg), following hydroxylapatite chromatography, was chromatographed on an Affi-Gel blue gel column as described under Methods. Four-milliliter fractions were collected at a flow rate of 0.4 mL/min. Each fraction was assayed for aminoacylation activity, and the absorbance was measured at 280 nm.

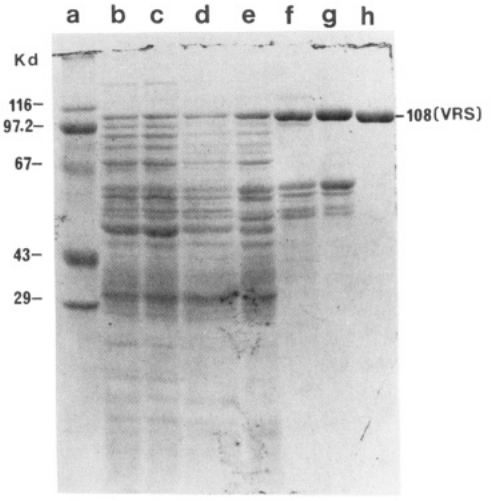

FIGURE 2: SDS–PAGE at each stage of the purification of valyl-tRNA synthetase. (a) Standard proteins: β-galactosidase (MW 116 000); phosphorylase *b* (MW 97 200); bovine serum albumin (MW 67 000); ovalbumin (MW 43 000); and carbonic anhydrase (MW 29 000). (b) Crude extract (54 μg); (c) autolysate (58 μg); (d) 45% ammonium sulfate supernatant (50 μg); (e) 60% ammonium sulfate precipitate (50 μg); (f) Sepharose 4B pool (19 μg); (g) hydroxylapatite pool (16 μg); (h) Affi-Gel blue gel pool (7 μg).

In the final step of its purification, VRS from the overproducing strain elutes as a single symmetrical peak from Affi-Gel blue gel, between 0.3 and 0.5 M NaCl, after unbound protein is washed from the column (Figure 1). The enzyme preparation contains a single protein band (M_r = 108 000) as determined by SDS–PAGE (Figure 2); a similar molecular weight is obtained by gel filtration under nondenaturing condition (results not shown). These values agree well with the molecular size inferred from the DNA sequence of the VRS gene (Heck & Hatfield, 1988; Hartlein et al., 1987) and are quite similar to the molecular weight of 110 000 determined

Table I: Amino Acid Composition of Valyl-tRNA Synthetase

amino acid	mol %[a] purified VRS	mol %[a] DNA sequence[b]	amino acid	mol %[a] purified VRS	mol %[a] DNA sequence[b]
Asp + Asn	11.8	11.7	Pro	4.6	4.7
Glu + Gln	12.8	12.4	Tyr	4.1	3.2
Ser	3.8	3.8	Val	6.5	6.3
Gly	8.3	7.2	Met	1.7	3.4
His	1.6	1.7	Ile	6.7	6.9
Arg	6.4	6.8	Leu	8.8	8.3
Thr	5.9	5.5	Phe	3.6	3.8
Ala	9.0	8.6	Lys	5.0	5.8

[a] Calculation is based on the mole percent of each amino acid, excluding cysteine and tryptophan, which were not determined in this analysis. [b] Heck and Hatfield (1988).

for the K12 enzyme by zonal and analytical ultracentrifugation (Yaniv & Gros, 1969).

Purified VRS is free of contaminating ribonuclease activity. *E. coli* $tRNA^{Val}$, incubated with the enzyme overnight at room temperature, retains 86% of its valine acceptance activity and shows little degradation when examined by polyacrylamide/8 M urea gel electrophoresis (results not shown).

The amino acid composition of VRS isolated from *E. coli* GRB 238/pHOV1 is in good agreement with that deduced from the DNA sequence of the VRS gene (Heck & Hatfield, 1988; Hartlein et al., 1987) (Table I). The low value for methionine results from the oxidation of methionine to methionine sulfoxide during hydrolysis of the protein in the vapor phase; methionine sulfoxide was not determined in our analysis. These results, however, differ considerably from the amino acid composition previously reported for the *E. coli* K12 enzyme (Yaniv & Gros, 1969); the reasons for this discrepency are not clear. The N-terminal sequence of our VRS preparation, Met-Glu-Lys-Thr-Tyr-Asn-Pro-Gln-Asp-Ile, is in perfect agreement with the sequence expected from the proposed translational start of the *E. coli* ValS gene (Heck & Hatfield, 1988).

Titration of free sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959) shows that three SH groups react in native VRS and five SH groups are reactive in the enzyme denatured with 8 M urea (data not shown). Yaniv and Gros (1969) reported two SH groups in native and five to six such groups in denatured VRS.

The steady-state kinetic parameters for purified VRS were determined by varying the concentrations of $tRNA^{Val}$ at fixed concentrations of ATP and valine chosen to be 3–5 times their K_M. No substrate inhibition by either ATP or valine was detected under the assay conditions used. At 25 °C, apparent K_M values of the VRS for both in vitro transcribed normal and FUra-substituted $tRNA^{Val}$ are approximately the same: 1.7×10^{-6} M for $tRNA^{Val}$ and 1.5×10^{-6} M for FUra-substituted $tRNA^{Val}$. These figures compare to the value of 0.6×10^{-6} M, at 30 °C, reported by Yaniv and Gros (1969), although they are 10 times higher than those we previously determined with native normal and FUra-substituted $tRNA^{Val}$ using a crude S100 cell extract (Horowitz et al., 1974). The V_{max} of 6.3–6.6 μmol (mg of protein)$^{-1}$ min^{-1} for both normal and (FUra)$tRNA^{Val}$ in the present experiments is about twice as great as that reported by Yaniv and Gros (1969).

^{19}F NMR Spectra of (FUra)$tRNA^{Val}$. Because of the resolution obtainable in ^{19}F NMR spectra and the sensitivity of the fluorine nucleus to changes in its environment, we have used ^{19}F NMR spectroscopy to probe conformational changes in (FUra)$tRNA^{Val}$ resulting from its interaction with VRS. At 47 °C, in 50 mM sodium cacodylate buffer, pH 6.0, containing 15 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA, 14

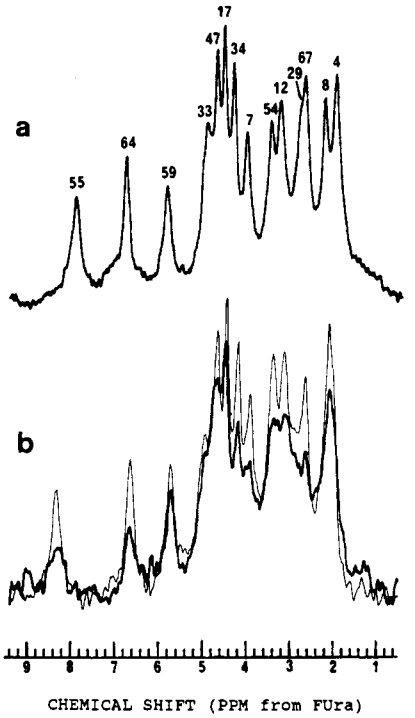

FIGURE 3: ^{19}F NMR spectra of 5-fluorouracil-substituted *E. coli* $tRNA^{Val}$. (a) Spectrum of (FUra)$tRNA^{Val}$ recorded at 47 °C in 50 mM sodium cacodylate buffer, pH 6.0, containing 15 mM $MgCl_2$, 100mM NaCl, and 1 mM EDTA. (b) Spectrum of free (FUra)-$tRNA^{Val}$ (thin line) and the (FUra)$tRNA^{Val}$–valyl-tRNA synthetase complex after addition of 0.5 equiv of VRS (thick line), recorded at 30 °C in the same buffer used in (a), except for 10 mM NaCl. Resonance assignments (Chu et al., submitted for publication) are indicated in (a).

resonances, including 1 shoulder, are resolved in the ^{19}F spectrum of fluorinated $tRNA^{Val}$ (Figure 3a), 1 for every incorporated FUra residue (see Figure 4). Spectra were routinely recorded at pH 6.0 to minimize chemical shift changes due to ionization of the N(3)–H of incorporated 5-fluorouracil. 5-Fluorouridine has a pK_a of 7.57 (Wempen et al., 1961), and titratable FUra residues in (FUra)$tRNA^{Val}$ have a somewhat higher pK_a value, ca. 8 (Hardin et al., 1986) [compare to values of 8.1–8.3 reported for FUra in poly(FU) by Szer and Shugar (1963) and Massoulie et al. (1963)].

We have recently assigned each of the signals in the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ by specific substitution for individual FUra residues of in vitro transcripts (Chu & Horowitz, 1989; Chu et al., submitted for publication). The results, indicated in Figure 3a, permit identification of the sites on the tRNA molecule affected by VRS binding. To minimize protein denaturation and because synthetases generally have a higher affinity for tRNA at a lower ionic strength (Bonnet & Ebel, 1975), the interaction of (FUra)$tRNA^{Val}$ and VRS was examined at 10 mM NaCl and 30 °C. Lowering the NaCl concentration and temperature induces changes in the ^{19}F NMR spectrum of the tRNA (compare Figure 3a and Figure 3b). As expected, the furthest downfield peak (FU55), whose chemical shift is very sensitive to salt concentration (Hardin et al., 1986), shifts downfield. FU34, FU12, and FU8 shift slightly upfield, with the latter now substantially overlapping the resonance assigned to FU4. FU29, which overlaps FU67 at 47 °C, shifts downfield and now appears either as a peak between FU12 and FU67 (Figure 3) or as an upfield shoulder on FU12. Despite these shifts, assignment of the spectrum at 10 mM NaCl and 30 °C remains unambiguous.

In many spectra, especially those recorded at low ionic strength or Mg^{2+} concentration, a fractional peak is noted 0.4

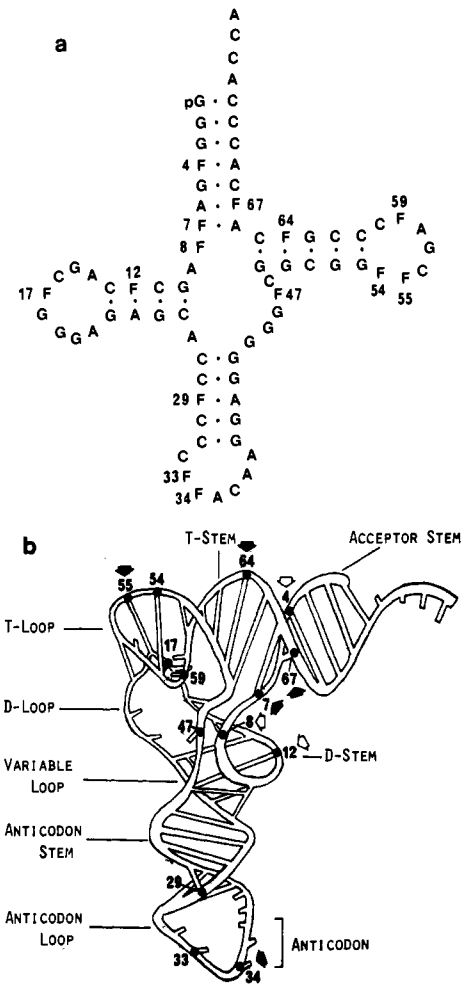

FIGURE 4: (a) Cloverleaf structure of *E. coli* $tRNA^{Val}$ with uracil and uracil-derived bases replaced by 5-fluorouracil (F). (b) Tertiary structure of $tRNA^{Val}$ based on the structure of yeast $tRNA^{Phe}$ (Kim et al., 1974). Positions of the 14 fluorouracils serving as ^{19}F NMR probes are indicated by filled circles and are labeled by residue number. 5-Fluorouracil residues interacting strongly with VRS are marked with solid arrows; positions showing moderate effects of VRS binding are indicated by open arrows.

ppm downfield of the signal from FU64 (Figure 3). This peak arises from a splitting of the resonance assigned to FU64, which is base-paired to G50 in the T-stem of (FUra)$tRNA^{Val}$. As the Mg^{2+} concentration is lowered, an increasing fraction of FU64 intensity shifts from 6.8 to 7.2 ppm (results not shown). Detection of separate resonances demonstrates that FU64 is in slow exchange, on the NMR time scale, between two microenvironments, presumably as a result of a magnesium-dependent conformational change involving the T-stem of the tRNA.

^{19}F Spectral Properties of the (FUra)$tRNA^{Val}$ Complex with VRS. Valyl-tRNA synthetase causes marked perturbations in the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$. Comparison of the spectrum of free and protein-bound (FUra)-$tRNA^{Val}$ (Figure 3b) shows line broadening and the decrease in peak intensity expected as a result of the longer motional correlation time of the tRNA–VRS complex. These changes cannot be ascribed to nonspecific effects of added protein because the ^{19}F NMR spectrum remains unaffected by addition of bovine serum albumin (results not shown).

The effects of VRS are selective; some resonances exhibit greater broadening and intensity losses than others. This can readily be seen in Figure 3b and, in greater detail, in Figure 5, which shows the effects on the ^{19}F spectrum of (FUra)-$tRNA^{Val}$ of adding increasing amounts of enzyme. Among

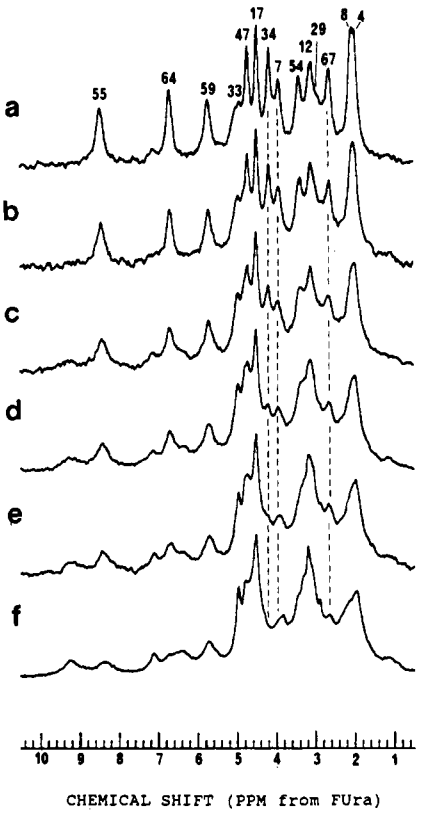

FIGURE 5: ^{19}F NMR spectra of the complex of 5-fluorouracil-substituted $tRNA^{Val}$ with valyl-tRNA synthetase (VRS). Spectra recorded in the absence of VRS (a) and in the presence of VRS at a molar ratio to tRNA of (b) 0.25, (c) 0.5, (d) 0.67, (e) 0.75, and (f) 1.00.

the most notable changes is a loss of intensity of FU34 (4.24 ppm), FU7 (3.96 ppm), and FU67 (2.67 ppm) as the concentration of VRS increases (indicated by dashed lines in Figure 5), with the resonance assigned to FU34, at the wobble position of the anticodon, showing the largest decrease. The loss of intensity at these resonances may be due to a gradual peak broadening caused by binding of the protein, or could result from a slow exchange of these fluorines between free and protein-bound environments with the position of the shifted peaks difficult to identify because of poor peak resolution; shoulders are observed on several ^{19}F resonances at high VRS concentrations (Figure 5).

Valyl-tRNA synthetase also alters the environment of FU55 and FU64, the two furthest downfield peaks in the spectrum of (FUra)$tRNA^{Val}$ (Figure 5), causing large chemical shift changes in the frequencies of these resonances. As synthetase is added, an increasing fraction of FU55 intensity undergoes a 0.8–0.9 ppm downfield change in chemical shift while FU64 intensity shifts upfield 0.3–0.4 ppm (Figure 5); both resonances also exhibit some additional line broadening. Detection of separate, broadened resonances suggests that these fluorines may be in slow or intermediate exchange between free and protein-bound states.

At higher VRS/tRNA ratios, broadening and shifting of resonances in the 2.9–3.4 ppm region of the spectrum occur. FU12 shifts slightly downfield, resulting in a better resolution of FU29 (2.9 ppm), and two shoulders appear in the region of the now overlapping resonances of FU12 and FU54 (Figure 5). The upfield peak, assigned to the poorly resolved resonances of FU8 and FU4, also broadens and exhibits shifts of intensity to lower field (Figure 5).

Solvent Accessibility of 5-Fluorouracils in the (FUra)-$tRNA^{Val}$ Complex with VRS. When interpreting the ^{19}F

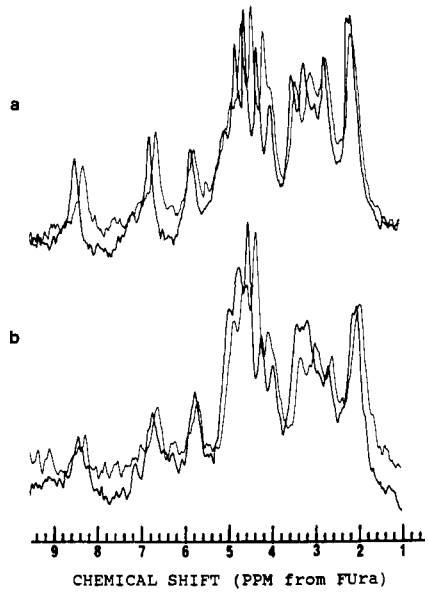

FIGURE 6: Solvent isotope shift of (a) free 5-fluorouracil-substituted $tRNA^{Val}$ and (b) the (FUra)$tRNA^{Val}$–valyl-tRNA synthetase complex after addition of 0.6 equiv of VRS. Thick lines are in 10% D_2O; thin lines are in 100% D_2O.

NMR spectral changes observed on VRS binding to (FUra)$tRNA^{Val}$, it is difficult to distinguish effects resulting from direct protein contact with a FUra base from those due to enzyme-induced long-range conformational changes in the tRNA. To help differentiate these possibilities, we have probed the solvent exposure directly at each fluorine nucleus in the tRNA. Because of an H–D isotope effect, ^{19}F resonances from FUra residues accessible to solvent shift to higher field when the solvent is changed from H_2O to D_2O (Hull & Sykes, 1976). This solvent isotope shift (SIS) provides a quantitative estimate of the degree of solvent exposure of individual FUra residues in (FUra)$tRNA^{Val}$ (Hardin et al., 1986). Figure 6 shows the chemical shift changes in the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ on switching solvent from 10% D_2O (thick lines) to 100% D_2O (thin lines) for free tRNA (Figure 6a) and for tRNA complexed with VRS at a molar ratio of enzyme to tRNA of 0.6 to 1 (Figure 6b). The expected upfield shift is readily discernible, and the SIS values are summarized in Table II. In the absence of synthetase, the SIS ranges from 0.04 to 0.18, compared to a value of 0.15 for free 5-fluorouridine, indicating that some fluorines are completely exposed whereas others make little or no contact with solvent (see Discussion). Upon VRS binding to (FUra)$tRNA^{Val}$, significant increases occur in the SIS of FU4/FU8 (poorly resolved resonances), FU54, and FU67 (Table II), suggesting that these fluorines may become more exposed to solvent in the VRS–tRNA complex. The solvent accessibility of the remaining 10 fluorouracils in (FUra)$tRNA^{Val}$ does not change.

Table II: Solvent Isotope Shifts (SIS) for Resonances in the ^{19}F NMR Spectra of FUra-Substituted $tRNA^{Val}$ Free and Complexed with VRS

fluorouracil residue	position in $tRNA^{Val}$	SIS[a] free $tRNA^{Val}$	SIS[a] $tRNA^{Val}$–VRS complex
FU4[b]	acceptor stem	0.06	**0.14**
FU7	acceptor stem	0.04	0.03
FU8[b]	tertiary	0.07	**0.15**
FU12	D-stem; tertiary	0.14	0.19
FU17	D-loop	0.18	0.17
FU29	anticodon stem	0.15	ND[c]
FU33	anticodon loop	0.16	0.14
FU34	anticodon	0.17	0.16
FU47	variable loop	0.15	0.16
FU54	T-loop; tertiary	0.09	**0.13**
FU55	T-loop; tertiary	0.17	0.15
FU59	T-loop	0.05	0.06
FU64	T-stem; GU wobble	0.13	0.13
FU67	acceptor stem	0.04	**0.09**
5-fluorouridine		0.15	

[a] SIS = δ(10% D_2O) − δ(100% D_2O) and is given in ppm. Boldface type indicates significant differences from free $tRNA^{Val}$. [b] Resonances FU4 and FU8 are not well resolved. [c] Not determined.

Discussion

To overcome difficulties in obtaining sufficient quantities of valyl-tRNA synthetase for our studies, VRS was purified from an overproducing strain of *E. coli*. We found that the amino acid content of this enzyme differs from that previously published for VRS isolated from *E. coli* K12 (Yaniv & Gros, 1969). The amino acid composition reported here compares well with that derived from the DNA sequence of the *E. coli* VRS gene (Table I). Furthermore, the N-terminal sequence of the purified enzyme agrees with that deduced from the VRS gene sequence (Heck & Hatfield, 1988; Hartlein et al., 1987) and provides the first experimental support for the predicted site of protein synthesis initiation in the VRS gene sequence (Heck & Hatfield, 1988).

Studies of the interaction of VRS with 5-fluorouracil-labeled *E. coli* $tRNA^{Val}$ described here illustrate the ability of ^{19}F NMR to provide information about tRNA recognition by its cognate aminoacyl-tRNA synthetase. The 14 FUra residues in (FUra)$tRNA^{Val}$ are distributed throughout all the stems and loops of the molecule (Figure 4) and serve as site-specific reporters of conformational changes induced by synthetase binding. Changes in the ^{19}F chemical shift position or the intensity of individual resonances reflect differences in the environment of the fluorine nucleus in the free and the protein-bound state.

Major changes in the ^{19}F NMR spectrum as VRS is added to (FUra)$tRNA^{Val}$ include a gradual decrease in the intensity of resonances corresponding to FU34 at the 5′ position of the anticodon, and to FU7 and FU67 at the base of the acceptor stem (Figure 5); the largest effect is on FU34. Higher concentrations of VRS produce broadening and downfield shifts of resonances FU12 and FU4/FU8 (Figure 5); the latter are not well resolved, and it is difficult to determine whether one or both resonances are affected. These FUra residues (with the exception of FU4) are all situated along the bend on the inside of the L-shaped tRNA molecule extending from the acceptor stem to the anticodon (Figure 4b). Presumably, the identity elements of (FUra)$tRNA^{Val}$ are located in this region of the molecule. Valyl-tRNA synthetase binding has little effect on FU17, FU29, FU33, FU47, and FU59, which are situated on the opposite side of the tRNA. These observations agree with the general model of tRNA–synthetase interaction advanced by Rich and Schimmel (1977), which suggests that synthetases bind their cognate tRNAs along and around the inside of the L-shaped structure, recognizing the acceptor stem and, depending on the particular enzyme and tRNA, possibly also the D-stem and the anticodon. Results of the high-resolution crystal structure determination of the *E. coli* glutaminyl-tRNA synthetase–$tRNA^{Gln}$–ATP complex (Rould et al., 1989) also lend support to this model.

Interaction of valyl-tRNA synthetase (from *Bacillus stearothermophilus*) with *E. coli* $tRNA^{Val}$ was studied by Schweizer and co-workers (Schweizer et al., 1984, 1989) using ^{13}C NMR of [4,5-^{13}C]uracil-labeled tRNA; only resonances

from the five modified uridines, 4-thiouridine-8, dihydrouridine-17, uridine-5-oxyacetic acid-34, ribothymidine-54, and pseudouridine-55, were assigned and monitored in these experiments. In agreement with our findings, these investigators reported that the largest spectral change on synthetase binding to the tRNA is at the wobble base, V34; lesser effects were found at positions s^4U8, rT54, and $\psi 55$. Because of better resolution and complete assignment of the ^{19}F NMR spectrum of (FUra)tRNA^{Val}, our results extend these observations to other parts of the tRNA molecule.

Observed spectral shifts caused by VRS binding to (FUra)tRNA^{Val} may be the result of either direct contact with a fluorine nucleus or an enzyme-induced, long-range structural change in the tRNA. Experiments to differentiate these possibilities by comparing the accessibility of individual nucleotides to chemical and enzymatic probes in the presence and absence of VRS are underway. Some, if not all, of the spectral changes are undoubtedly due to direct contact of the enzyme with a FUra residue. This may be especially true for FU34 in the anticodon of *E. coli* tRNA^{Val}. The anticodon is known to be an essential recognition element of this tRNA; VRS is able to discriminate between tRNAs on the basis of their anticodon sequence (Schulman & Pelka, 1988). Our results (Chu and Horowitz, unpublished results) indicate that position 36, at the 3′ end of the anticodon, has the most important role: substitution of A, G, or U for C36 decreases k_{cat}/K_M by 2–3 orders of magnitude. Substitutions for A35 also greatly diminish the activity of tRNA^{Val}. Position 34 plays a distinct but lesser part; replacing U34 with C lowers k_{cat}/K_M 70%.

Direct contact of VRS with FU8 is consistent with proposals suggesting that a covalent bond between U8 of tRNA and aminoacyl-tRNA synthetases is a common intermediate in the aminoacylation reaction, at least in prokaryotes (Schoemaker & Schimmel, 1977; Koontz & Schimmel, 1979; Starzyk et al., 1982). Inactivation of synthetases by 5-bromouridine, and the synthetase-catalyzed exchange reaction between solvent and hydrogen at carbon-5 of uracil-8, suggested formation of a Michael adduct by nucleophilic attack of an enzyme SH group on the 6-position of U8 in tRNA. However, no evidence for such an adduct is observed in our ^{19}F NMR experiments. Saturation of the 5,6-double bond of 5-fluorouracil by reaction with sodium bisulfite (Sander & Deyrup, 1972; Hardin et al., 1986), by reduction to form fluorodihydrouracil (Horowitz et al., 1983), and by formation of binary and ternary complexes of 5-fluorodeoxyuridylate and thymidylate synthetase (Byrd et al., 1978; Lewis et al., 1980, 1981) results in a 35–40 ppm upfield shift of the ^{19}F signal. Formation of a stable Michael adduct between VRS and FU8 of (FUra)tRNA^{Val} should produce a similar upfield shift of the resonance of FU8, but no new peak was observed in the upfield region of the ^{19}F NMR spectra of (FUra)tRNA^{Val}–VRS complexes (results not shown). Furthermore, several tRNA^{Val} variants having U8 replaced by other bases retain appreciable, although reduced, levels of amino acid accepting activity (Chu et al., submitted for publication), which suggests that U8 is important but not essential for aminoacylation of this tRNA.

In addition to the ^{19}F spectral effects already described, VRS binding results in large chemical shift changes for the resonances of FU55 and FU64, both located in the T-arm of (FUra)tRNA^{Val}; resonance FU55 moves downfield while FU64 shifts upfield. Two components are visible for both resonances (Figure 5), indicating slow exchange. The direction of chemical shift change is difficult to interpret in terms of changes in tRNA structure. We have found that ^{19}F signals from FUra residues in Watson–Crick base pairs resonate at higher field than do those from 5-fluorouracils in single-stranded regions of tRNA (Hardin et al., 1986, 1988; Chu & Horowitz, 1989). Signals from FUra base-paired to G (wobble pairing), however, resonate downfield of those from 5-fluorouracils in single-stranded loops (Chu & Horowitz, 1989). Thermal denaturation of (FUra)tRNA^{Val} results in a downfield shift of the ^{19}F resonances from 5-fluorouracils in FU-A base pairs whereas the fluorine signal of FUra base-paired to G shifts upfield (Hardin et al., 1986). The upfield shift of the signal from the wobble-paired FU64 on VRS binding may thus be due to a partial unwinding of the T-stem helix.

The downfield shift of resonance FU55 resulting from VRS interaction with (FUra)tRNA^{Val} may also be indicative of a move of FU55 to a less hydrophobic, more exposed, environment. Although in ^{19}F NMR studies with fluoroamino acid labeled proteins downfield shifts have been correlated with fluorines in a hydrophobic (interior) environment (Hull & Sykes, 1974; Robertson et al., 1977), Metzler and Lu (1989), in their ^{19}F NMR investigation of cro repressor binding to a 5-fluorodeoxyuridine-labeled 17 base pair O_R3 operator sequence, observed upfield shifts of ^{19}F resonances from fluorines involved in hydrophobic interactions with amino acid side chains of the repressor. Furthermore, on the basis of an investigation of inclusion complexes of substituted fluorobenzenes with β-cyclodextrins, Hansen et al. (1985) also concluded that large upfield chemical shift changes result from placing the ^{19}F nucleus in a more hydrophobic environment. Peak broadening and the low intensity of the shifted FU55 and FU64 resonances make it difficult to measure the SIS of the shifted peaks to confirm the greater solvent accessibility of the fluorines of FU55 and FU64 in the VRS–tRNA^{Val} complex. If our interpretation of the structural basis for the chemical shifts of resonances FU55 and FU64 is correct, the ^{19}F NMR results imply conformational changes involving the T-loop and stem of (FUra)tRNA^{Val} on VRS binding, possibly a (partial) disruption of T-loop/D-loop interactions.

Solvent isotope shift experiments indicate that four FUra residues—FU4 and FU67 in the acceptor stem, FU54 in the T-loop, and FU8—become more exposed to solvent as a result of VRS binding to (FUra)tRNA^{Val} (Table II). These results must be interpreted with caution, however, because of the relatively poor resolution of the ^{19}F spectrum of the enzyme–tRNA complex. The increased SIS values might be due to peaks shifted by VRS binding that underly resonances from free tRNA.

Examination of the SIS values for the FUra residues in free (FUra)tRNA^{Val} provides several interesting insights. As expected, fluorouracils located in the loop regions of the tRNA, FU17 (D-loop), FU33 and FU34 (anticodon loop), and FU47 (variable loop), are highly solvent-accessible. FU59 in the T-loop, however, is shielded from solvent. This is not an unexpected result. U59 in yeast tRNA^{Phe} is known to be protected from reaction with a variety of chemical reagents specific for pyrimidines in single-stranded regions [reviewed by Goddard (1977)], presumably because it is stacked on U60 and on the C48-G15 base pair (Quigley & Rich, 1976); FU59 in (FUra)tRNA^{Val} fails to react with bisulfite (Hardin et al., 1986). 5-Fluorouracil residues in the acceptor stem, FU4, FU7, and FU67, have low SIS values (0.04–0.06) compared to that of free fluorouridine under the same conditions (0.15), which indicates that these fluorines are highly shielded from solvent. Surprisingly, three FUra's situated in tRNA stems are quite solvent-accessible: FU29 in the anticodon stem has

an SIS value comparable to that of free FUrd; the same is true for FU12 in the D-stem, which is part of a base triple with A9 and A23, and for FU64, which makes a wobble pair with G50 in the T-stem. Comparison with SIS values reported earlier for native (FUra)tRNA^{Val} isolated from FUra-treated *E. coli* (Hardin et al., 1986) shows that FU64 in the in vitro transcript is more highly solvent-exposed. By contrast, FU67 at the base of the acceptor stem is highly shielded in the in vitro transcript but is partially exposed to solvent in native (FUra)tRNA^{Val} (Hardin et al., 1986). These differences presumably reflect the small structural differences between tRNAs transcribed in vitro and those isolated from bacterial cells (Sampson & Uhlenbeck, 1988; Chu & Horowitz, 1989).

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Sequence-Specific ^{1}H NMR Assignments and Structural Characterization of Bovine Seminal Fluid Protein PDC-109 Domain b†

Keith L. Constantine,‡ Vasudevan Ramesh,‡,§ László Bányai,∥ Maria Trexler,∥ László Patthy,∥ and Miguel Llinás*,‡

Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, and Institute of Enzymology, Biological Research Center, 1502 Budapest, Hungary

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ABSTRACT: Sequence-specific resonance assignments for the isolated second or b domain of the bovine seminal fluid protein PDC-109 have been obtained from analysis of two-dimensional ^{1}H NMR experiments recorded at 500 MHz. These assignments include the identification of all aromatic and most aliphatic amino acid resonances. Stereospecific assignment of resonances stemming from the Val^2 $CH_3^{\gamma,\gamma'}$ groups and from seven $CH^{\beta,\beta'}$ geminal pairs has been accomplished by analysis of $^3J_{\alpha\beta}$ coupling constants in conjunction with patterns of cross-peak intensities observed in two-dimensional nuclear Overhauser effect (NOESY) spectra. Analysis of NOESY and $^3J_{\alpha NH}$ data reveals a small antiparallel β-sheet involving stretches containing residues 25–28 and 39–42, a *cis*-proline residue (Pro^4), antiparallel strands consisting of residues 1–3, 5–7, and 10–13, and an aromatic cluster composed of Tyr^7, Trp^{26}, and Tyr^{33}. The results of distance geometry and restrained molecular dynamics calculations indicate that the global fold of the PDC-109 b domain, a type II module related to those found in fibronectin, is somewhat different from that predicted by modeling the structure on the basis of homology between type II and kringle units. A shallow depression in the molecular surface which presents a solvent-exposed hydrophobic area—a potential ligand-binding site—is identified in the NMR-based models.

PDC-109 is a major protein component of bovine seminal plasma (Esch et al., 1983). Analysis of the primary structure revealed that PDC-109 consists of two homologous domains, a and b, of 38 and 41 amino acid residues, respectively (Esch et al., 1983). The domains are characterized by a double cystine polypeptide loop pattern distinguished by disulfide bridges connecting Cys residue pairs 1–3 and 2–4. The PDC-109 modules are members of a family of domains collectively known as type II structures (Petersen et al., 1989). Two collinear type II repeats are present in both of the chains of fibronectin (Petersen et al., 1983; Skorstengaard et al., 1984; Kornblihtt et al., 1985). Type II domains are also found as single units in blood coagulation factor XII (McMullen & Fujikawa, 1985) and insulin-like growth factor receptor II (Morgan et al., 1987), as two tandem domains in the bovine seminal plasma protein BSP-A3 (Seidah et al., 1987), and as three head-to-tail repeats in the 72- and 92-kDa type IV collagenases (Collier et al., 1988; Wilhelm et al., 1989). In addition, a remote homology has been identified between the ~40 amino acid, two-disulfide type II domains and the ~80 amino acid, three-disulfide kringle structures found in blood clotting and fibrinolytic proteins (Patthy et al., 1984). In the absence of experimental data, this homology was used to generate molecular models of various type II domains from the X-ray crystallographic structure of bovine prothrombin kringle 1 (Holland et al., 1987).

The physiological roles of PDC-109 are under active investigation. PDC-109 has been observed to stimulate the release of gonadotropins when added to cultures of pituitary cells (Manjunath & Sairam, 1987), and there is evidence that sperm motility is affected by PDC-109 binding (Manjunath et al., 1987). PDC-109 has recently been shown to interact with apolipoprotein A-I in vitro (Manjunath et al., 1989). Fibronectin-like antigens in human seminal plasma (Vuento et al., 1980) are likely to be closely related to PDC-109. The functionally better characterized type II domains are those found in fibronectin, which are unique to the gelatin (denatured collagen) binding region. Thus, the type II domains are believed to be involved in fibronectin/collagen interactions (Yamada, 1989). It is also noteworthy that PDC-109 can be readily purified on a gelatin-agarose column (Manjunath et al., 1987), directly demonstrating gelatin binding mediated by type II domains.

Structural studies of isolated type II domains are crucial if the physiological roles of these modules are to be understood in molecular detail. Here we report the results of two-dimensional (2D)[1] ^{1}H NMR studies of a fragment of PDC-109,

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‡ Carnegie Mellon University.
§ Current address: Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.
∥ Institute of Enzymology.

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