Protein Science (2000), *9*:2573–2576. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Fluorescence and ^{19}F NMR evidence that phenylalanine, 3-L-fluorophenylalanine and 4-L-fluorophenylalanine bind to the L-leucine specific receptor of *Escherichia coli*

LINDA A. LUCK AND COLIN JOHNSON

Department of Chemistry, Clarkson University, Potsdam, New York 13699

(RECEIVED May 9, 2000; FINAL REVISION August 7, 2000; ACCEPTED September 22, 2000)

Abstract: The binding capacity of the L-leucine receptor from *Escherichia coli* was measured with L-phenylalanine and 4-fluoro-L-phenylalanine as substrates by fluorescence. The apparent dissociation constants (K_D) for L-leucine, L-phenylalanine, and 4-fluoro-L-phenylalanine are 0.40, 0.18, and 0.26 respectively. ^{19}F NMR data show protein-induced shifts for the 4-fluoro-L-phenylalanine peak and 3-fluoro-L-phenylalanine when receptor is present. Evidence points to the binding of only the L-isomers of these fluorine analogs.

Keywords: ^{19}F NMR; fluorescence; 3-fluoro-phenylalanine; 4-fluoro-phenylalanine; leucine; periplasmic proteins; receptor

Receptors for amino acids are members of a family of binding proteins found in the periplasm of Gram-negative bacteria. These proteins are essential for the uptake of a wide variety of sugars, amino acids, peptides, oxyanions, and other nutrients (Adams & Oxender, 1989). Receptors initiate the transport of these various substances by recognizing and specifically binding to them in the periplasm. Upon binding a conformational change that takes place in the receptor protein (Olah et al., 1993; Quiocho & Ledvina, 1996). This change allows recognition of the substrate-bound receptor to the transport system and subsequent deposit of the substrate into the cytoplasm of the bacteria (Antonucci & Oxender, 1988). Ligand-induced conformational change mechanism of these receptors is also implicated in the process of chemotaxis (Luck & Falke, 1991).

Much information has been gleamed from the study of these receptors and their binding modes. One system in particular that has been of interest has been the leucine transport system, which is known to transport hydrophobic amino acids. Two leucine-binding proteins with overlapping specificity for the branched-chain amino acids are present in *Escherichia coli.* The two proteins, leucine-isoleucine-valine binding protein (LIV) and the leucine specific protein (LS), utilize the same set of membrane-bound components to transport leucine across the membrane. LIV has been shown to bind leucine, valine, isoleucine, and to a lesser extent, threonine, serine, and alanine, whereas LS was shown to bind leucine specifically as well as a fluorinated analog 5′,5′,5′-trifluoroleucine (Adams & Oxender, 1989).

The membrane components have been shown to be homologous to the cystic fibrosis (CF) transmembrane conductance regulator (CFTR). It was shown that the prokaryotic transport system, LIV-I, is functionally similar to the CFTR protein (Gibson et al., 1991). The LIV receptor has also shown homology to the group 1 metabotropic glutamate receptors (Costantino et al., 1999) and the *N*-methyl-D-aspartate receptor (Masuko et al., 1999) illustrating the importance of investigating this prokaryotic complex as a model for other important systems.

To date, the tertiary structures of LS and LIV have been solved in the open form and the open-unliganded form (Sack et al., 1989a, 1989b). This has precluded any investigation of the binding mode of hydrophobic substances in these receptors and the reasons for the difference in the binding specificity of the proteins. Our laboratory has taken on the investigation of the two proteins to try to understand how hydrophobic substances bind in the ligand pocket and the reason for the discrimination among the branched-amino acid substrates. In our ongoing interrogation, we have found by ^{19}F NMR and fluorescence studies that the LS also binds L-phenylalanine, 4-fluoro-L-phenylalanine, and 3-fluoro-L-phenylalanine. These data have open the possibilities that these receptors are more promiscuous in their binding abilities, and they may be useful in the investigations of binding modes of fluorinated substrates such as those used in medical imaging.

Results and discussion: Our laboratory has been interested in the binding modes for hydrophobic ligands, and in our initial studies we incorporated 5-fluorotryptophan into the LS protein to address ligand binding in solution NMR and solid state NMR (Luck et al., unpubl. obs.). During our studies, we found that our internal standard for ^{19}F NMR, 4-fluorophenylalanine, bound to the LS protein. To this end, we further investigated the binding capacity of LS for fluorophenylalanines by ^{19}F NMR.

Reprint requests to: Linda A. Luck, Department of Chemistry, Clarkson University, Potsdam, New York 13699; e-mail: luckla@clarkson.edu.

The usefulness of ^{19}F NMR as a probe for binding of ligands stems from the rather unique properties of the nucleus. This spin 1/2 center has high NMR sensitivity, and the resonances are easily resolved and assigned because the chemical shift range is up to two orders of magnitude larger than that of proton. The fluorine lone pair electrons, which provide a large paramagnetic term in the shielding formula, exhibit a strong environmental dependence and will detect local structural changes. Aromatic fluorine atoms are particularly sensitive to changes in electronic density changes of the adjacent π system. When a fluorinated ligand binds to a protein, the fluorine atom experiences a protein induced chemical shift, which arises from the change in environment, experienced by the fluorine as it leaves the aqueous phase and enters the hydrophobic binding site. This protein-induced chemical shift is manifested as a broadened peak at higher frequency in the NMR spectrum than the peak of the free fluorinated ligand in solution. Such shifts have been found in the ^{19}F NMR spectra of fluorodeoxy sugars bound to the glucose and galactose binding protein of *E. coli* (Luck, 1995).

Figure 1 shows the ^{19}F NMR spectra of LS with 4-fluoro-D/L-phenylalanine (top trace) and excess leucine (bottom trace). In the top spectrum, there is a sharp peak for the 4-fluoro-D/L-phenylalanine at −40 ppm and a broadened peak at −35 ppm that represents the 4-fluoro-L-phenylalanine in the binding pocket of the LS protein. When an excess of L-leucine is added to the NMR sample, the peak at −35 ppm disappears as leucine displaces the 4-fluoro-L-phenylalanine from the binding pocket. In addition, the integrated area of the peak at −40 ppm increases indicating 4-fluoro-L-phenylalanine has left the hydrophobic binding pocket and is in the solution. To further investigate this finding, we used 4-fluoro-D-phenylalanine in the NMR experiment. No protein-induced chemical shift was observed in the spectrum. By these NMR data, we conclude that LS is specific for the L-isomer of this fluorophenylalanine.

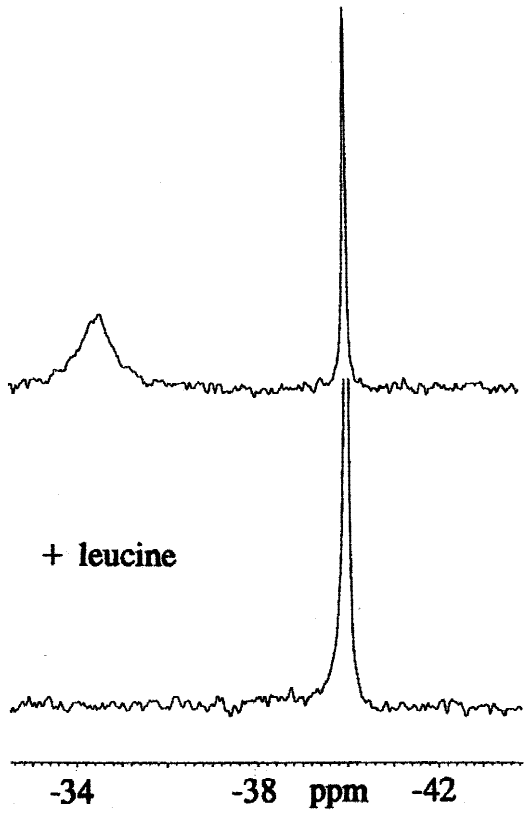

Fig. 1. The 470 MHz ^{19}F NMR spectra of LS and 4-fluoro-phenylalanine. Top trace shows a peak at −34 ppm representing the 4-fluoro-L-phenylalanine in the binding pocket. The free 4-fluoro-D-phenylalanine and excess 4-fluoro-L-phenylalanine is shown as a narrow peak at −40 ppm. The bottom trace shows the receptor with a 10-fold excess of L-leucine added. The peak at −34 ppm has disappeared as leucine displaced the 4-fluoro-L-phenylalanine in the ligand-binding site.

A protein-induced chemical shift was also observed in the spectrum of LS and 3-fluoro-D/L-phenylalanine. In this spectrum (not shown), the peak for the free ligand was found at −38 ppm, and a protein-induced chemical shift was observed at −37 ppm. Because the D or L only isomer was not commercially available, we were unable to test for specificity of this ligand in either the NMR or fluorescence experiments. As in the case of the 4-fluoro-D/L-phenylalanine, a peak remained at −38 ppm even when excess protein was used in the experiment. These NMR data indicate that the fluoro-phenylalanines bind to the LS protein, which has not been reported in the literature.

To corroborate this finding, we subjected LS to a fluorescence-binding assay in which we used 4-fluoro-L-phenylalanine and 4-fluoro-D-phenylalanine in separate experiments. Figure 2 shows the data for the binding assay showing the K_D of 0.26 μM for 4-fluoro-L-phenylalanine. We have illustrated that LS does not bind 4-fluoro-D-phenylalanine by this assay. Table 1 also includes the fluorescence binding data and K_Ds for other ligands. It is important to note that our fluorescence assay shows the same results for the binding of L-leucine (K_D of 0.40 μM) as published in the literature (Adams et al., 1991). We have also included L-methionine as a negative control.

Our data have shown by NMR and fluorescence that the L-leucine specific receptor from *E. coli* is not as specific as reported in the literature. We have shown that 4-fluoro-L-phenylalanine binds to LS with a K_D of 0.26 μM, and L-phenylalanine with a K_D of 0.18 μM. We have shown by NMR that LS binds 3-fluoro-L-phenylalanine. From our data, we can also conclude that only the L form of the ligands bind to LS. The authors hypothesize that the study of the binding pocket of LS may hold the key to unanswered questions about the specificity of hydrophobic ligands in receptors and binding modes for fluorinated aromatic substrates. This also raises important questions as to the specificity of other periplasmic binding proteins and their ability to transport substances that may not be important for viability. Our laboratory is further investigating LS and its ability to bind to a multitude of ligands, and we are also investigating the binding pocket using multidimensional NMR methods.

Materials and methods: All materials were used in the purest form. The fluorinated amino acids were purchased from Sigma Chemical Company (St. Louis, Missouri).

Production of the LS protein: For the production of the L-leucine binding protein large quantities of the receptor were obtained by expression of the plasmid pKSty, which was obtained from Dr. Dale Oxender (Adams et al., 1991). For preparation of this protein, bacterial cells were grown in large batch cultures of Lurial Broth,

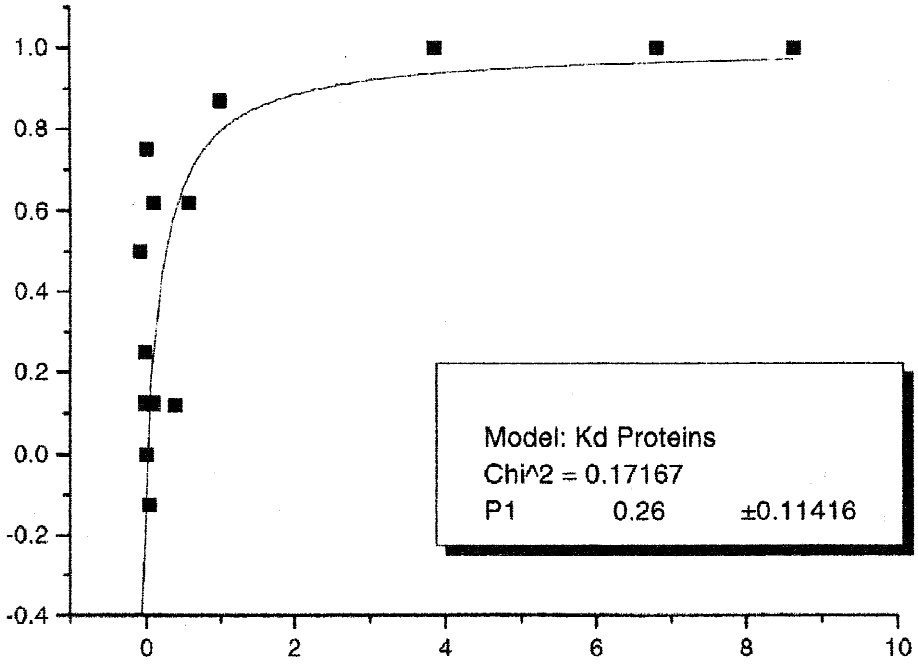

Fig. 2. The fluorescence binding assay of LS and 4-fluoro-L-phenylalanine. The Y-axis represents the fraction of the ligand bound and the X-axis represents the fraction of the ligand free. K_D is measure in μM.

to which was added 100 μg/mL ampicillin. After growth overnight the cultures were inoculated with 0.5 mM IPTG and were harvested 4 h after this induction. Protein was obtained by osmotic shock methods and purified by DEAE sepharose with a linear gradient of 0–0.5 M NaCl. Proteins were dialyzed against 6 L of phosphate buffer to reduce the salt concentration.

Fluorescence binding measurements: Ligand binding to the substrate site was monitored by the resulting increase in tryptophan fluorescence according to a published procedure (Boos et al., 1972). Ligand was titrated into each sample, and the resulting fluorescence changes were normalized to an identical reference sample titrated with substrate-free buffer. Fluorescence measurements used excitation at 285 nm and emission at 338 nm with 5 nm bandwidths and a 3 s response time. Sample components contained 150 nM protein, 150 nM NaCl, 10 mM Tris, pH 7.1 at 25 °C.

Table 1. *Fluorescence binding assay*

Ligand	K_D (μM)
L-Leucine	0.40
4-Fluoro-L-phenylalanine	0.26
4-Fluoro-D-phenylalanine	>1 mM
L-Phenylalanine	0.18
D-Phenylalanine	>1 mM
L-Methionine	>1 mM

NMR measurements: ^{19}F NMR spectra were obtained at 470 MHz on a Varian 500 spectrometer with a 5 mm $^{19}F/^{1}H$ probe tuned to the ^{19}F resonance. Samples of L-leucine binding protein (0.5 mM) in 10 mM phosphate buffer, pH 7.0 were prepared with 10% D_2O (v/v) as the lock solvent. ^{19}F resonances were referenced to trifluoroacetic acid as an external standard at 0 ppm. Standard decoupled parameters included temperature at 25 °C, relaxation delay of 0.7 s, weep width of 12,000 Hz, and 25 Hz line broadening.

Acknowledgments: Acknowledgment is made to the Donors of The Petroleum Research Fund, administered by the American Chemical Society for partial support of this research. We also thank Dale Oxender for the LS constructs.

References

Adams MD, Maguire DJ, Oxender DL. 1991. Altering the binding activity and specificity of the leucine binding proteins of *Escherichia coli*. *J Biol Chem 266*:6209–6241.

Adams MD, Oxender DL. 1989. Bacterial periplasmic binding protein tertiary structures. *J Biol Chem 264*:15739–15742.

Antonucci TK, Oxender DL. 1988. Transport of branched-chain amino acids in *Escherichia coli*. *Methods Enzymol 166*:245–251.

Boos W, Gordon AS, Hall RE, Price HD. 1972. Transport properties of the galactose-binding protein of *Escherichia coli* substrate-induced conformational changes. *J Biol Chem 247*:917–924.

Costantino G, Macchiarulo A, Pellicciari R. 1999. Modeling of amino-terminal domains of group I metabotropic glutamate receptors: Structural motifs affecting ligand selectivity. *J Med Chem 42*:5390–5401.

Gibson AL, Wagner LM, Collins FS, Oxender DL. 1991. A bacterial system for investigating transport effects of cystic fibrosis associated mutations. *Science 254*:109–111.

Luck LA. 1995. ^{19}F NMR studies of fluorinated sugars binding to the glucose and galactose receptor. In: Crabb JW, ed. *Techniques in protein chemistry VI*. San Diego: Academic Press. pp 487–494.

Luck LA, Falke JJ. 1991. ^{19}F NMR studies of the D-galactose chemosensory

receptor. 1. Sugar binding yields a global structural change. *Biochemistry 30*:4248–4256.

Masuko T, Kashiwagi K, Kuno T, Nguyen ND, Pahk AJ, Fukuchi JI, Igarashi K, Williams K. 1999. A regulatory domain (R1–R2) in the amino terminus of the *N*-methyl-D-aspartate receptor: Effects of spermine, protons, and ifeprodil and the structural similarity to bacterial leucine/isoleucine/valine binding protein. *Mol Pharmacol 55*:957–969.

Olah GA, Trakhanov S, Trewhella J, Quiocho FA. 1993. Leucine/isoleucine/valine-binding protein contracts upon binding of ligand. *J Biol Chem 268*:16241–16247.

Quiocho FA, Ledvina PS. 1996. Atomic structure and specificity of bacterial perplasmic receptors for active transport and chemotaxis: Variation of common themes. *Mol Microbiol 20*:17–25.

Sack JS, Saper MA, Quiocho FA. 1989a. Periplasmic binding protein structure and function. Refined X-ray structures of the leucine/isoleucine/valine-binding protein and its complex with leucine. *J Mol Biol 206*:171–191.

Sack JS, Trakhanov SD, Tsigannik IH, Quiocho FA. 1989b. Structure of the L-leucine-binding protein refined at 2.4 Å resolution and comparison with the Leu/Ile/Val-binding protein structure. *J Mol Biol 206*:193–207.