Site-Directed Spin Labeling Demonstrates That Transmembrane Domain XII in the Lactose Permease of *Escherichia coli* Is an α-Helix†

John Voss,‡ Molly M. He,‡ Wayne L. Hubbell,§ and H. Ronald Kaback*,‡

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, and Jules Stein Eye Institute and Department of Chemistry & Biochemistry, University of California, Los Angeles, California 90095-1662

Received April 11, 1996; Revised Manuscript Received June 11, 1996⊗

ABSTRACT: Functional lactose permease mutants containing single-Cys residues at positions 387−402 [He, M. M., Sun, J., & Kaback, H. R. (1996) *Biochemistry 35,* 12909−12914] and a biotin acceptor domain in the middle cytoplasmic loop were solubilized in *n*-dodecyl-β-D-maltopyranoside and purified by avidin affinity chromatography. Each mutant protein was derivatized with a thiol-selective nitroxide reagent and examined by conventional and power saturation electron paramagnetic resonance spectroscopy. Analysis of the electron paramagnetic resonance spectral line shapes and the influence of O_2 on the saturation behavior of the spin-labeled proteins were measured in order to obtain information on the mobility of the spin-labeled side chains and their accessibility to O_2, respectively. The data show a periodic dependence of both mobility and accessibility on sequence position consistent with an α-helical structure. These results provide direct support for the contention that transmembrane domain XII is in an α-helical conformation and on the periphery of the 12-helix bundle that comprises the lactose permease molecule.

Site-directed spin labeling (SDSL)[1] has proven to be a particularly valuable technique for investigating membrane protein structure and dynamics [reviewed by Hubbell and Altenbach (1994a,b)]. The method involves introduction of a single-Cys residue into a protein by site-directed mutagenesis, followed by labeling with a thiol-specific reagent containing a nitroxide spin-label. SDSL of proteins has been used to study the secondary structure and/or dynamics of bacteriorhodopsin (Altenbach et al., 1989, 1990, 1994; Greenhalgh et al., 1991) and visual rhodopsin (Farahbakhsh et al., 1993, 1995; Resek et al., 1993), to measure distances between two paramagnetic centers (Anthony-Cahill et al., 1992; Rabenstein & Shin, 1995; Voss et al., 1995a,b), to provide the electrostatic potential at specific sites (Shin & Hubbell, 1992), and to identify sites of tertiary interaction (Hubbell & Altenbach, 1994a; Mchaourab et al., 1996).

In the preceding paper (He et al, 1996), Cys-scanning mutagenesis of transmembrane domain XII in the lactose (lac) permease demonstrates that none of the residues is essential for activity. Moreover, none of the single-Cys-replacement mutants in this domain is inactivated by alkylation with N-ethylmaleimide. In this communication, SDSL is used to study single-Cys replacement mutants in transmembrane domain XII. On the basis of the periodicity of side chain mobility and accessibility to molecular O_2, it is concluded that the residues in this domain are arranged along an α-helix with one face in contact with the protein and the other in contact with the hydrocarbon chains of the bilayer.

EXPERIMENTAL PROCEDURES

Materials

(1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (methanethiosulfonate spin-label) was a gift from Kálmán Hideg and is available from Reanal (Budapest, Hungary). Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. All restriction endonucleases, T_4 DNA ligase, and $Vent_R^{TM}$ DNA polymerase were from New England Biolabs, Beverly, MA. Sequenase was from United States Biochemical, Cleveland, OH. All other materials were reagent grade and obtained from commercial sources.

Methods

Construction, Expression, and Purification of Mutant Lac Permeases. In order to facilitate avidin affinity purification, the biotin acceptor domain from a *Klebsiella pneumoniae* oxaloacetate decarboxylase was inserted into the middle cytoplasmic loop of single-Cys mutants at positions 387−402 (Consler et al., 1993); the single-Cys mutant at position 400 was not included because L400C[2] permease is poorly expressed (He et al., 1996). After the desired mutations were confirmed by dideoxynucleotide sequence analysis (Sanger et al., 1977; Hattori & Sakaki, 1986), *Escherichia coli* T184 (*lacZ*$^-$*Y*$^-$) was transformed with plasmid encoding a given mutant. Cells were grown aerobically at 37 °C in LB broth with streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Dense cultures were diluted 10-fold in a 12 L fermentor and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After growth for another 2 h at 37 °C, cells were harvested and disrupted by passage through a French pressure cell. A membrane

† The research reported here was supported in part by NIH Grant EY05216 to W.L.H., and the Jules Stein Professorship Endowment.

* To whom correspondence and reprint requests should be addressed.

‡ Departments of Physiology and Microbiology & Molecular Genetics.

§ Jules Stein Eye Institute and Department of Chemistry & Biochemistry.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: SDSL, site-directed spin labeling; lac, lactose; EPR, electron paramagnetic resonance; DPPH, 2,2-diphenyl-1-picrylhydrazyl; KP_i, potassium phosphate; PCR, polymerase chain reaction; DM, *n*-dodecyl-β-D-maltopyranoside.

[2] Site-directed mutants are designated by the one-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in the wild-type lac permease, followed by a second letter indicating the amino acid replacement.

S0006-2960(96)00877-X CCC: $12.00

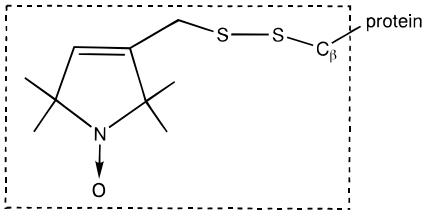


FIGURE 1: Structure of nitroxide side chain R1.

fraction was isolated by centrifugation and extracted with 3% DM, and permease was purified by affinity chromatography on immobilized monomeric avidin as described (Wu et al., 1994). Each purified mutant protein was then incubated with 100 μM (1-oxyl-2,2,5,5-tetramethyl) methanethiosulfonate for 30 min at room temperature to generate the nitroxide side chain R1 (Figure 1), concentrated, and dialyzed using a Micro-ProDiCon membrane (Spectrum) (Voss et al., 1995b).

EPR Spectroscopy. EPR measurements on given spin-labeled lac permease mutants contained in TPX capillaries were performed at 22 °C. Five to ten microliters of sample containing purified permease at a final concentration of ca. 50−80 μM in 10 mM MES (pH 7.5)/0.02% DM was used in each measurement. Spectra were obtained using a Varian E-109 X-band spectrometer fitted with a loop-gap resonator (Froncisz & Hyde, 1982; Hubbell et al., 1987) at a microwave power of 2 mW and a modulation amplitude of 4 G and recorded under field-frequency lock at X-band. Signal-averaged spectra (eight scans) were obtained with a 100 G sweep at 30 s/scan using a Nicolet 1280 computer.

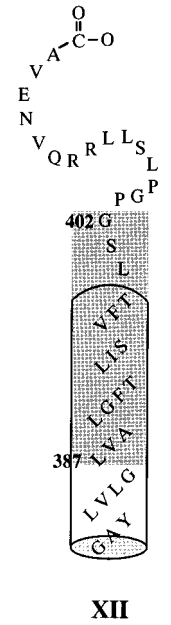
Power saturation measurements were carried out as previously described (Altenbach et al., 1989) in a N_2 atmosphere and in the presence of O_2 in equilibrium with air and analyzed in terms of the parameter $P_{1/2}$, which is proportional to $1/T_{1e}T_{2e}^3$ (Altenbach et al., 1989). $P_{1/2}$ is the microwave power which saturates the signal amplitude to $^1/_2$ the value it would have reached in the absence of saturation. Spin exchange with a fast-relaxing paramagnetic species such as O_2 results in an increase in the relaxation rate ($1/T_{1e}$) in proportion to the collision frequency (Altenbach et al., 1989). The change in $P_{1/2}$ due to the presence of O_2, $\Delta P_{1/2} = P_{1/2}(O_2) - P_{1/2}(N_2)$, is a direct measure of the collision frequency of the nitroxide with O_2. To account for the effect of T_{2e} and variations in spectrometer performance, $\Delta P_{1/2}$ is normalized to give the "accessibility parameter", Π, defined as

$$\Pi = \frac{\Delta P_{1/2}(O_2)}{P_{1/2}(\mathrm{DPPH})} \frac{\Delta H(\mathrm{DPPH})}{\Delta H}$$

where $P_{1/2}$(DPPH) is $P_{1/2}$ for a 2,2-diphenyl-1-picrylhydrazyl (DPPH) crystal, ΔH(DPPH) is the peak-to-peak line width of the DPPH resonance, and ΔH is the corresponding line width of the nitroxide central ($m_I = 0$) resonance (Farahbakhsh et al., 1992).

RESULTS

EPR of Spin-Labeled Single-Cys Permeases. EPR spectra of the R1 nitroxide side chain at positions 387−402 (excluding position 400) in transmembrane domain XII of lac permease are shown in Figure 3. The inverse of the line width of the central resonance, $1/\Delta H$, is an increasing function of the motional freedom[4] of the nitroxide in its local environment (Mchaourab et al., 1996). A plot of $1/\Delta H$ versus the position of the R1 side chains in transmembrane domain XII demonstrates that nitroxides at positions 388,



XII

FIGURE 2: Secondary structure model of transmembrane domain XII in lac permease. The one-letter amino acid code is used, and the residues in domain XII studied by SDSL are shaded.

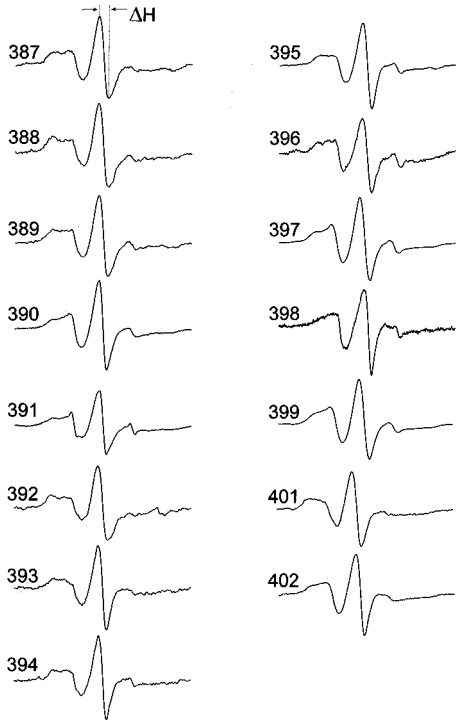


FIGURE 3: First-derivative EPR spectra of given nitroxide-labeled single-Cys lac permease mutants. The spectra are scaled vertically for convenience of presentation, and the magnetic field scan width is 98 G. The line width of the central resonance (ΔH) is indicated on the spectrum of residue 387.

[3] T_{1e} is the spin−lattice relaxation time; T_{2e} is the transverse relaxation time.

[4] The term motional freedom is used in a general sense, and a change in motional freedom can arise from a change in either the rate or amplitude of motion, or both.

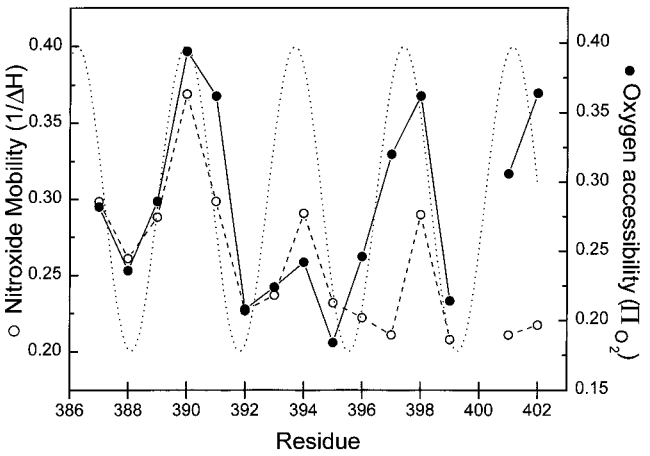


FIGURE 4: $\Pi(O_2)$ (solid line) and $1/\Delta H$ (broken line) versus sequence position for the nitroxide-labeled single-Cys residues at positions 387−402 in lac permease. The dotted curve is that for a function of period 3.6, and comparison with the $\Pi(O_2)$ and $1/\Delta H$ functions confirms that the data are consistent with an α-helical structure.

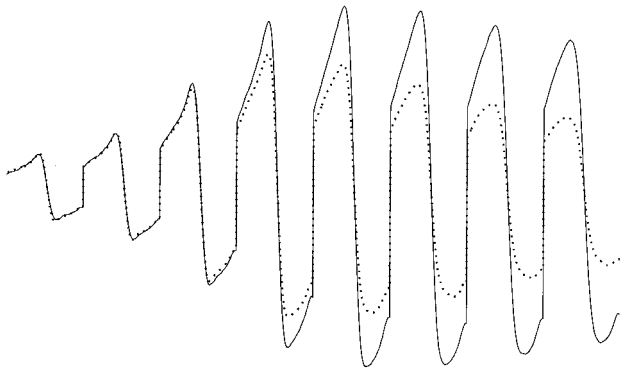


FIGURE 5: Saturation behavior for spin-labeled lac permease at position 390 (L390R1) in a N_2 atmosphere (dotted line) and in the presence of O_2 (solid line). Shown from left to right are 10 G scans of the center line at 0.1, 0.25, 1, 4, 9, 16, 25, and 36 mW. At high microwave powers, the presence of O_2 leads to larger amplitudes due to the decreased T_{1e}.

392, 395, and 399 are highly constrained by this measure (Figure 4, open symbols). Clearly, there is a periodicity in the mobility of the residues in this domain that likely reflects tertiary contacts of the nitroxide side chains at these positions with other parts of the permease. Moreover, the periodicity observed is consistent with an α-helix in which the residues on one face are more immobilized than those on the other.

Accessibility Analysis. Another approach used in topological mapping is examination of the collision rate of R1 with diffusible paramagnetic species (Hubbell & Altenbach, 1994a). As described in Methods, $\Delta P_{1/2}$ is determined from the saturation behavior of the central resonance ($m_I = 0$) line in the absence and presence of a paramagnetic relaxing agent such as O_2. As an example of the data, Figure 5 shows a series of magnetic field scans of the $m_I = 0$ resonance for increasing microwave power levels in a N_2 atmosphere (dotted line) or in the presence of molecular O_2 in equilibrium with air (solid line). Relaxation due to the presence of paramagnetic O_2 is evident in the larger signal amplitudes at higher microwave powers.

The accessibility parameter Π is derived from $\Delta P_{1/2}$ normalized to the intrinsic line width of R1 and the saturation behavior of a DPPH standard. Figure 4 (filled symbols) shows $\Pi(O_2)$ as a function of R1 position in transmembrane domain XII of the permease. The data display a profile virtually identical to that observed for nitroxide mobility (i.e. $1/\Delta H$), with the same periodicity. Clearly, these findings are also consistent with the contention that transmembrane domain XII is α-helical.

DISCUSSION

As demonstrated previously using spectral second moments (Hubbell & Altenbach, 1994a), the mobility of nitroxide side chains in a scanned region of a protein may exhibit a periodicity indicative of a specific secondary structure. For example, a transmembrane α-helix may have two faces, one on which the residues are relatively immobile because they are sterically constrained by interactions with other portions of the protein and the other face on which the residues are more mobile because they are exposed to the lipid hydrocarbon chains. In this case, a periodicity of approximately 3.6 is expected. In contrast, with a β-sheet structure under the same conditions, a periodicity of 2 is expected.

The dynamics reported by the attached nitroxide may include contributions from the rotational diffusion of the whole protein, the motion of the side chain relative to the backbone, and the motion of the backbone relative to the average structure. Since the rotational diffusion of the permease in a DM micelle is too slow to affect the conventional EPR line shape, motional narrowing arises from the latter two types of motion. However, in a region of continuous secondary structure, the variability in backbone dynamics will be relatively small from one residue to the next, while the variability in side chain dynamics may be dramatic due to interactions with the milieu surrounding the protein (Mchaourab et al., 1996). On the basis of the outer splittings, which resolve individual dynamic components and the amplitude of each component, most of the spectra appear heterogeneous. In heterogeneous spectra, the center line width is dominated by the motion of the most mobile component. Thus, a spin-labeled side chain that samples two environments, partially buried or solvent-exposed, is classified as surface-accessible according to $1/\Delta H$ analysis. The $1/\Delta H$ parameter is therefore very useful in characterizing side chains that lack a highly mobile population, and the variations in $1/\Delta H$ are attributed to periodic changes in tertiary contacts that arise from a helical structure which interacts anisotropically with the remainder of the protein.

The data of Figure 4 clearly reveal periodic changes in $\Pi(O_2)$ that are in phase with those of $1/\Delta H$, although the relative amplitudes of the oscillations in $\Pi(O_2)$ are different. There is a divergence in the $1/\Delta H$ to $\Pi(O_2)$ correlation at residues 396 and 397. In particular, the $1/\Delta H$ parameter at position 397 is not in phase with the periodicity function plotted in Figure 4 (dotted line). This divergence may be due to a bulky neighboring side chain (L400 or L394) which could limit the range of motion of 397R1, while O_2 accessibility from the other direction remains unhindered. As defined, the "accessibility" of a nitroxide to O_2 is a function of both local steric constraints imposed by the protein structure and the product of the local diffusion constant and concentration, $D[O_2]$ (Altenbach et al., 1989). It is not possible to predict $D[O_2]$ in the micellar interior, and variations in this product may contribute to variations in the amplitude of the maxima in $\Pi(O_2)$.

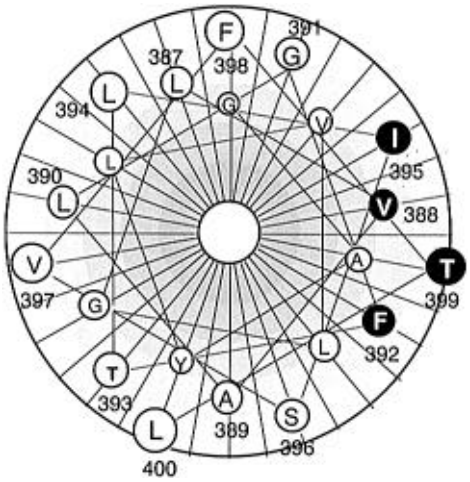


FIGURE 6: Helical wheel representation of transmembrane domain XII of the lactose permease. Numbered residues were investigated by site-directed spin labeling. Residues in contact with other protein structure are highlighted by filled circles.

An ideal helical wheel representation of the transmembrane domain XII is shown in Figure 6. Residues corresponding to minima in Figure 4 with respect to $1/\Delta H$ and $\Pi(O_2)$ (low mobility and accessibility) are indicated with solid circles. It is apparent that the data are entirely consistent with a helical structure for transmembrane domain XII in which approximately 300° of the helix is solvated by the fluid interior of the bilayer (open circles) and a surface of approximately 60° interfaces with another transmembrane domain(s) (solid circles).

It is particularly noteworthy that each of the 15 Cys-replacement mutants studied displays normal transport activity, even after exposure to *N*-ethylmaleimide (He et al., 1996). Therefore, it is likely that the mutants retain near native structure even with the attached spin-label. Although addition of the nitroxide side chain must induce at least some perturbation in the backbone and the position of the side chain, it is unlikely that the effects are of sufficient magnitude to alter the overall interpretation that transmembrane domain XII is an α-helix [see Hubbell and Altenbach (1994b) and Mchaourab et al. (1996)]. It is also relevant that, in these studies, the permease is embedded in DM micelles rather than in a phospholipid bilayer. It should be emphasized, however, that previous studies utilizing site-directed fluorescence (Jung et al., 1994; Wu & Kaback, 1994), introduction of engineered metal binding sites (i.e. bis- or tris-His residues; Jung et al., 1995; He et al., 1995a,b), and a monoclonal antibody that binds to a conformational epitope (Sun et al., 1996) indicate that the permease retains near native structure under these conditions.

In conclusion, therefore, the studies presented here provide strong evidence that transmembrane domain XII in the lac permease is in an α-helical conformation.

ACKNOWLEDGMENT

We thank Christian Altenbach and Lukasz Salwinski for assistance with data fitting and Kerstin Stemple for synthesizing deoxyoligonucleotide primers.

REFERENCES

Altenbach, C., Flitsch, S. L., Khorana, H. G., & Hubbell, W. L. (1989) *Biochemistry 28*, 7806−7812.

Altenbach, C., Marti, T., Khorana, H. G., & Hubbell, W. L. (1990) *Science 248*, 1088−1092.

Altenbach, C., Greenhalgh, D. A., Khorana, H. G., & Hubbell, W. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1667−1671.

Anthony-Cahill, S. J., Benfield, P. A., Fairman, R., Wasserman, Z. R., Brenner, S. L., Stafford, W. d., Altenbach, C., Hubbell, W. L., & DeGrado, W. F. (1992) *Science 255*, 979−983.

Consler, T. G., Persson, B. L., Jung, H., Zen, K. H., Jung, K., Prive, G. G., Verner, G. E., & Kaback, H. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6964.

Cronan, J. E., Jr. (1990) *J. Biol. Chem. 265*, 10327−10333.

Farahbakhsh, Z. T., Altenbach, C., & Hubbell, W. L. (1992) *Photochem. Photobiol. 56*, 1019−1033.

Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G., & Hubbell, W. L. (1995) *Biochemistry 34*, 8812−8819.

Froncisz, W., & Hyde, J. S. (1982) *J. Magn. Reson. 47*, 515−521.

Ge, M., & Freed, J. H. (1993) *Biophys. J. 65*, 2106−2123.

Greenhalgh, D. A., Altenbach, C., Hubbell, W. L., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8626−8230.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 1291−1297.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Biochemistry 34*, 15667−15670.

He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 15661−15666.

He, M. M., Sun, J., & Kaback, H. R. (1996) *Biochemistry 35*, 12909−12914.

Hubbell, W. L., & Altenbach, C. (1994a) *Curr. Opin. Struct. Biol. 4*, 566−573.

Hubbell, W. L., & Altenbach, C. A. (1994b) in *Membrane protein structure* (White, S. H., Ed.) pp 224−248, Oxford University Press, New York.

Hubbell, W. L., Froncisz, W., & Hyde, J. S. (1987) *Rev. Sci. Instrum. 58*, 1879−1886.

Jung, H., Jung, K., & Kaback, H. R. (1994) *Protein Sci. 3*, 1052−1057.

Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 6272−6277.

Kaback, H. R. (1996) in *Handbook of Biological Physics II: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., & Lolkema, Eds.) Elsevier, Amsterdam (in press).

Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. (1993) *J. Bioenerg. Biomembr. 25*, 627−636.

Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Zen, K. (1994) *J. Exp. Biol. 196*, 183−195.

Likhtenshtein, G. I. (1993) *Biophysical labeling methods in molecular biology*, Cambridge University Press, New York.

Mchaourab, H., Lietzow, M. A., Hideg, K., & Hubbell, W. L. (1996) *Biochemistry* (in press).

McKenna, E., Hardy, D., Pastore, J. C., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2969−2973.

Rabenstein, M. R., & Shin, Y. K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 8239−8243.

Resek, J. F., Farahbakhsh, Z. T., Hubbell, W. L., & Khorana, H. G. (1993) *Biochemistry 32*, 12025−12032.

Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Shin, Y. K., & Hubbell, W. L. (1992) *Biophys. J. 61*, 1443−1453.

Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−998.

Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12300−12303.

Voss, J., Salwinski, L., Kaback, H. R., & Hubbell, W. L. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12295−12299.

Wu, J., & Kaback, H. R. (1994) *Biochemistry 33*, 12166−12171.

Wu, J., Frillingos, S., Voss, J., & Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.

Wu, J., Perrin, D., Sigman, D., & Kaback, H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

BI9608774