Biophysical studies of cytochromes B5 with amino acid substitutions in the membrane-binding domain

Alexey S. Ladokhin, V. G. Tretyachenko-Ladokhina, Peter W. Holloway, L. Wang,* and A. W. Steggles*
Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908; and *Department of Biochemistry, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272 USA

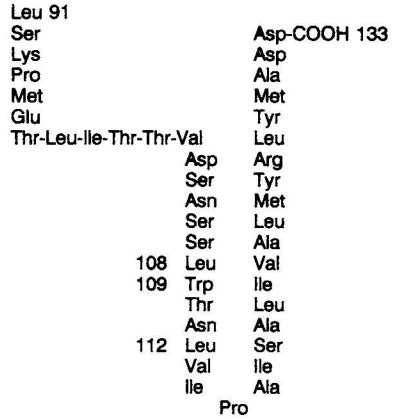
INTRODUCTION

Cytochrome b_5 (b_5), isolated by detergent extraction from liver (1), has been used extensively as a model membrane protein. The membrane-binding domain comprises approximately 40 amino acid residues and the protein binds rapidly and spontaneously to lipid vesicles. The protein contains three Trp (at 108, 109, and 112) in the membrane-binding domain and numerous studies have focussed on the fluorescent properties of these Trp (1–3). In addition, Fourier-transform infrared studies have been used to estimate the secondary structure and depth of penetration of this domain in the bilayer (4, 5). This protein has now been expressed in *Escherichia coli* and here we describe some studies with a mutant of rabbit liver b_5 where two of the Trp were replaced by Leu so that there now is only one Trp (109) in the membrane-binding domain (nonpolar peptide, NPP) (Scheme I) (6).

RESULTS AND DISCUSSION

These amino acid substitutions have not produced any gross changes in the structure of the membrane-binding domain of the b_5. Both the mutant and the native b_5 were bound to a series of lipid vesicles made from 1-palmitoyl-2(dibromostearoyl)-phosphatidylcholine, where the bromines are located at different positions of the sn-2 acyl chain (7). As shown in Fig. 1 the fluorescence quenching versus the depth of the bromine atoms was very similar for the two b_5's, which indicates that the depths in the membrane, of Trp 109 of the mutant b_5 and the "fluorescent Trps" (6) of the native b_5, are comparable. In addition, the position of the amide I band (at 1,655 cm^{-1}) in the FT-IR spectrum of lipid-bound NPP is indicative of a high content of α helix in both native and mutant NPP (Fig. 2). However the membrane-binding properties of the two b_5's are quite different. Fig. 3 shows the fluorescence enhancement when the mutant and normal b_5 are bound to 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) small unilamellar vesicles. Three conclusions can be drawn: the relative fluorescence enhancement seen with the mutant b_5 is greater than that seen with the native b_5. This is consistent with the depth of the single Trp 109 being somewhat greater than the average effective depth of the fluorescent Trps in the native b_5. This agrees with the bromolipid quenching data (Fig. 1). Second, the amount of lipid per b_5 needed to generate the maximum fluorescence (that ratio of lipid to b_5 which is needed to ensure all protein is bound) is greater with the mutant b_5 with the native b_5. This means that, at vesicle saturation, there will be fewer mutant b_5's bound per vesicle than normal b_5's bound per vesicle. Thirdly, the smaller curvature seen with the mutant compared to the native b_5 (comparing the data points to the straight lines which indicate infinite binding affinity) shows that the affinity of the mutant for the vesicle is less. The second and third observations are self consistent. If the affinity of the mutant for the vesicle is less, then repulsion between the negatively charged polar domains will dominate and so reduce the number of proteins which can saturate the vesicle.

The replacement of Trp by Leu is predicted by hydropathicity analysis (8) to increase the average hy-



SCHEME I

Address correspondence to Dr. Peter Holloway.

0006-3495/92/04/79/03 $2.00

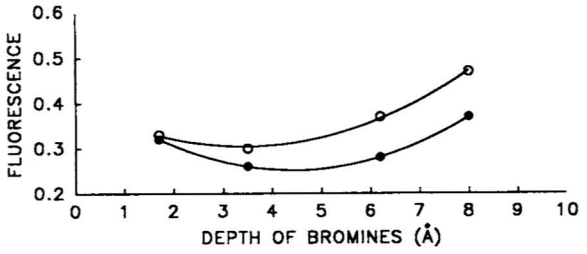


FIGURE 1 The relative fluorescence of b_5 in different brominated lipids. The relative fluorescence is plotted versus the average depth of bromine atoms below the head-group-hydrocarbon boundary (7). The fluorescence of native b_5 (○—○) and mutant b_5 (●—●) is normalized to the fluorescence in POPC for each curve separately.

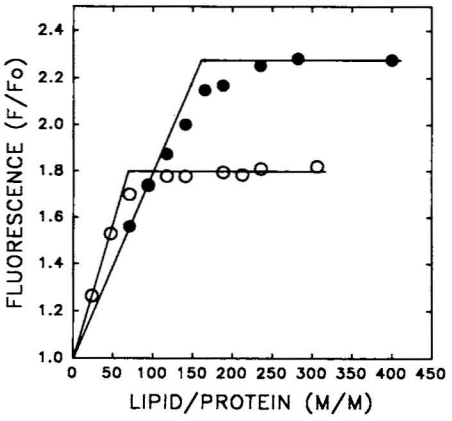


FIGURE 3 The fluorescence enhancement of native and mutant b_5. The proteins (1.7 μM) in buffer were mixed with aliquots of 6 mM POPC and the fluorescence at 340 nm was observed after excitation at 280 nm. Native b_5 (○—○) and mutant b_5 (●—●).

dropathicity of the NPP from 0.4 to 0.6 and so increase the affinity of the protein for lipid vesicles. This was not seen and suggests that some subtle changes in the conformation of the membrane-binding domain result from the Trp-to-Leu substitutions. This different conformation may result in a different interaction with the bilayer lipids and may be reflected in the disordering effect of this domain on the acyl chains of the bilayer. The temperature dependence of the position of the CH_2 symmetric stretching band in the infrared (Fig. 4) suggests that native NPP has a ordering effect on the acyl chains below the phase transition but very little effect above the phase transition. The mutant NPP has the same effect below the phase transition but also, after cooling, undergoes either a structural or topological change such that it then has a large ordering effect above the phase transition.

These results suggest that the membrane-binding domain of b_5, and perhaps other similar membrane-anchoring domains, has an amino acid sequence which generates a specific required secondary and tertiary structure. Small changes in primary sequence are sufficient to so alter this structure that there are profound changes in its interaction with lipid. Because of this sequence-structure relationship, we suggest it may be unwise to predict the structure of the membrane-binding domains of proteins based merely on the hydrophobicity of the amino acid residues.

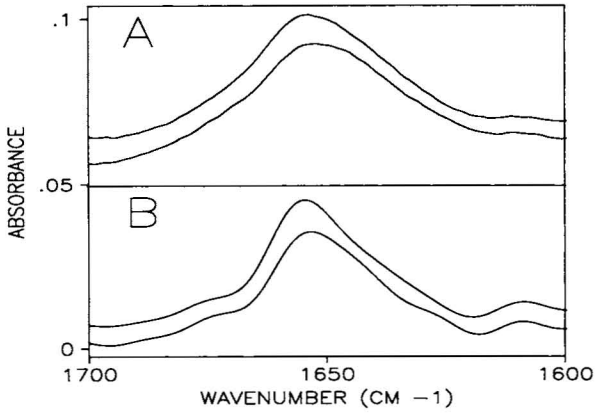


FIGURE 2 Infrared spectra of lipid bound native and mutant NPP in the region of the amide I band. Infrared spectra were recorded at 32°C and subjected to Fourier self-deconvolution as described previously (5). Samples (0.5 mM in protein) were prepared by adding the lipid vesicles (50 mM) to samples of the lyophilized NPP. (*A*) Spectra of native (*upper*) and mutant (*lower*). (*B*) Deconvolved spectra of native (*upper*) and mutant (*lower*).

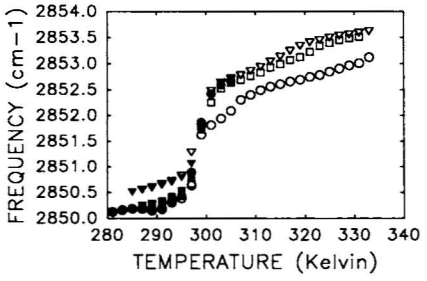


FIGURE 4 Effect of native and mutant NPP on the acyl chain order. Infrared spectra were collected as described in the legend to Fig. 2 and the center of mass of the band at ~2,850 cm^{-1} was determined. The samples were initially cooled from 305°K (*solid symbols*) and then heated up to 330°K (*open symbols*). (*Triangles*) DMPC alone, (*squares*) with native NPP, (*circles*) with mutant NPP.

Supported by GM 23858 and funds from the American Heart Association, Ohio Affiliate, and by the Ohio Board of Regents Academic and Research Challenge.

REFERENCES

1. Markello, T., A. Zlotnick, J. Everett, J. Tennyson, and P. W. Holloway. 1985. Determination of the topography of cytochrome b_5 in lipid vesicles by fluorescence quenching. *Biochemistry.* 24:2895–2901.
2. Fleming, P. J., D. E. Koppel, A. L. Y. Lau, and P. Strittmatter. 1979. Intramembrane position of the fluorescent tryptophanyl residue in membrane-bound cytochrome b_5. *Biochemistry.* 18:5458–5464.
3. Kleinfeld, A. M., and M. Lukacovic. 1985. Energy-transfer study of cytochrome b_5 using the anthroyloxy fatty acid membrane probes. *Biochemistry.* 24:1883–1890.
4. Holloway, P. W., and H. H. Mantsch. 1989. The structure of cytochrome b_5 in solution by fourier-transform infrared spectroscopy. *Biochemistry.* 28:931–935.
5. Holloway, P. W., and C. Buchheit. 1990. Topography of the membrane-binding domain of cytochrome b_5 in lipids by fourier transform infrared spectroscopy. *Biochemistry.* 29:9631–9637.
6. Ladokhin, A. S., L. Wang, A. W. Steggles, and P. W. Holloway. 1991. Fluorescence study of a mutant cytochrome b_5 with a single tryptophan in the membrane-binding domain. *Biochemistry.* 30: 10200–10206.
7. McIntosh, T. J., and P. W. Holloway. 1987. Determination of the depth of bromine atoms in bilayers formed from bromolipid probes. *Biochemistry.* 26:1783–1788.
8. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.