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Amino acid substitutions in the membrane-binding domain of cytochrome b_5 alter its membrane-binding properties

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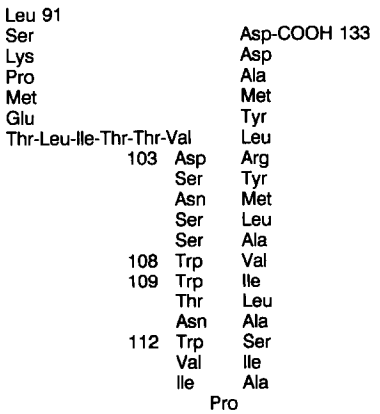
The structure–function relationships of the 43-amino-acid membrane-binding domain of cytochrome b_5 have been examined in two mutant forms of the protein. In one mutant, two tryptophans in the membrane-binding domain, at positions 108 and 112, were replaced by leucines, and in the second mutant, in addition, aspartic acid 103 was also replaced by leucine. The fluorescence emission spectra of the three proteins and their degree of quenching by brominated lipids indicate that the mutations are not producing major conformational changes or allowing a deeper degree of penetration of the domain into the bilayer. The hydrophobicities of the three proteins were compared, by determining strengths of self-association and membrane affinities, and it was found that the protein with two additional leucines was much less hydrophobic and the one with three additional leucines was much more hydrophobic than the native cytochrome. It appears that small changes in amino acid composition, which produce no gross changes in the structure of the membrane-binding domain, will nevertheless produce very large changes in the strengths of self- and membrane-association. These differences in self-association had profound effects on the times required for membrane-association to reach equilibrium.

Introduction

Cytochrome b_5 (b_5) is an integral membrane protein which was originally isolated from liver endoplasmic reticulum but has recently been expressed in and purified from *E. coli* [1] and yeast [2]. The protein contains 133 amino acid residues and its attachment to natural and artificial membranes is via a small 43 residue hydrophobic domain (nonpolar peptide, NPP, Scheme I). This laboratory has previously documented the self aggregatory properties of the isolated protein [3], the binding of the protein to membranes [4], and the exchange of the protein between membranes [5], all of these properties are governed by the hydrophobicity of the membrane-binding domain. Cytochrome b_5 contains three Trp in the membrane-binding domain (at positions 108, 109 and 112, Scheme I) and many of our earlier studies used the fluorescence properties of these residues to examine the protein. In our previous publi-



Scheme I.

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Abbreviations: b_5, cytochrome b_5, (the complete 133 amino acid residue protein); native b_5, the native cytochrome b_5 isolated from rabbit liver; (108, 112) mutant b_5, the cytochrome isolated from *E. coli* with Trp 108 and Trp 112 of the native sequence both replaced by Leu; (103, 108, 112) mutant b_5, the cytochrome isolated from *E. coli* with Asp 103, Trp 108 and Trp 112 of the native sequence all replaced by Leu; NPP, nonpolar peptide, the membrane-binding domain of the cytochrome b_5 which is released from cytochrome b_5 by trypsin treatment; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; BRPC, 1-palmitoyl-2(dibromostearoyl)phosphatidylcholine with bromine atoms at the 4,5-, 6,7-, 9,10- or 11,12 positions, as indicated.

cation [1] we showed that replacement of Trp's 108 and 112 by Leu had little effect on the topology of the NPP, as judged by these fluorescence properties, and in addition the secondary structure, as estimated by Fourier transform infrared spectroscopy, was unaffected [6]. The hairpin structure shown in Scheme I is supported by the work of Ozols [7] who was able to isolate a peptide corresponding to residues 91 through 127 by trypsin treatment of rabbit liver microsomal membranes. The structure shown in Scheme I, based on that of Ozols, places Asp-103 and Arg-127 at the membrane interface and Ozols suggested that removal of the Asp residue, as is seen in chicken b_5 with a Gln at this position, would result in a deeper penetration of the NPP into the membrane. We consequently generated a mutant of b_5 which has Asp-103 replaced by Leu with the expectation that the polypeptide chain would insert to place Glu-96 at the interface and so locate the single Trp-109 as much as 10 Å deeper in the membrane. To our surprise this alteration increased the depth of Trp-109 in the membrane by less than 1 Å and suggested that the topology of this small NPP in the membrane was not governed by such simple constraints. As the major role of the NPP in the native protein is to anchor the cytochrome to the endoplasmic reticulum, the effect of these substitutions upon the hydrophobicity of the whole protein was examined and it was found that these relatively minor amino acid replacements had a major effect upon this crucial membrane property. A preliminary report of these results has been made [8].

Materials and Methods

Rabbit liver cytochrome b_5 was isolated as described previously and the brominated lipids and small unilamellar sonicated lipid vesicles were prepared as described previously [9]. The two mutants (108, 112) and (103, 108, 112) were isolated from *E. coli* XL-1 cells (Stratagene, La Jolla, CA) which contain the vector pKK223–3 (Pharmacia, Piscataway, NJ) with an insert which carries the procaryote ribosome-binding site and the rabbit liver b_5 sequence [10] where the codons for Trps-108 and 112 and, in the second mutant Asp-103, have been replaced by codons for Leu, using the procedure of Kunkel [11]. All mutations were confirmed by DNA sequencing. The expression of the protein in *E. coli* and its method of isolation have been described [1] and the methods used were identical for the two mutants. Fluorescence measurements were made with an SLM 8000c spectrofluorometer (SLM/Aminco, Urbana, IL) as described previously [1].

Gel filtration was performed on a column of Sephadex G200 superfine (1 × 24 cm) equilibrated in 20 mM Tris acetate-0.2 mM EDTA (pH 8.1 at 20°C). Cytochrome samples were applied to the column in 0.12 ml of Tris acetate-EDTA which also contained 0.4 M KCl and 0.4 M sucrose. The column effluent was monitored at 412 nm and was collected in a burette. Possible binding sites on the column were first saturated by pre-running a sample containing 6.8 nmol of native b_5 and then the following samples were analyzed in succession: native b_5 6.8, 3.4 and 0.8 nmol; (108, 112) mutant b_5 6.8, 3.4 and 1.7 nmol; (103, 108, 112) mutant b_5 6.8, 3.4 and 1.7 nmol. The column was calibrated with blue dextran and potassium ferricyanide which eluted at 8.1 and 20.9 ml, respectively.

Binding of the b_5's to POPC vesicles was monitored by measuring the increase in Trp fluorescence upon addition of lipid vesicles, the precise procedure was slightly different with one of the mutants. With the native and (108, 112) mutant b_5 the following procedure was used. To a solution of protein (1.7 μM) in 10 mM Hepes-0.1 mM EDTA (pH 7.3 at 20°C) (Hepes buffer) were added aliquots of POPC vesicles (from a 6 mM stock solution) to give the required lipid/protein ratios. The samples were then incubated at 20°C for 30 min after each lipid addition before the fluorescence was measured. With the (103, 108, 112) mutant a modified procedure had to be used since the rate of binding is very slow (see below). Into each of eight cuvettes was placed a solution (0.7 μM) of (103, 108, 112) mutant b_5 in Hepes buffer. Baseline fluorescence and absorbance measurements were taken and then POPC vesicles (from a 6 mM stock solution) were added in increasing volumes to the different cuvettes to give the desired lipid/protein ratios. The cuvettes were incubated at 20°C for 16 h and the fluorescence was determined. All raw fluorescence values were corrected for dilution and inner filter effects and were then normalized to the maximum fluorescence value obtained with each protein at the highest lipid/protein ratio obtained.

The kinetics of binding and exchange of the different b_5's were obtained by adding small unilamellar POPC vesicles (final concentration 0.44 mM) as rapidly as possible to the different b_5's (2 μM) while the fluorescence was monitored at 340 nm and the solution was excited at 280 nm in a Perkin-Elmer MPF-44A fluorometer. When the maximum fluorescence increase was achieved, 6,7 BRPC (final concentration 0.44 mM) was added and the fluorescence monitored further. The experiment was then repeated with each protein with the first addition being BRPC and the second POPC.

Results

Characterization of the mutant cytochromes b_5

The purity of the isolated cytochromes was ascertained by measurement of the ratio of their absorbances at 412 nm vs. 280 nm. These ratios were 2.8

TABLE I

Position of fluorescence maxima and relative fluorescence in brominated lipids of native and mutant b_5's

Fluorescence was measured at 20°C with an SLM 8000c spectrofluorometer using an excitation wavelength of 293 nm to select only the Trp component. Lipid vesicles (final concentration 0.44 mM) were added to the protein (2 μM) in 10 mM Hepes-0.1 mM EDTA (pH 7.3 at 20°C) and the fluorescence was measured 60 minutes after mixing. The maxima of the emission spectra of the protein bound to POPC vesicles (λ_{max}) were calculated using a log-normal distribution of intensities [1]. The fluorescence of the proteins bound to BRPC vesicles was determined as described in Materials and Methods, and the relative fluorescence is the ratio of the area under the spectrum (for native and 108, 112) or the intensity measured at 335 nm (for 103, 108, 112) in the corresponding brominated lipid to the same parameter measured when the protein was bound to POPC.

b_5	λ_{max} (nm)	Relative fluorescence (%)			
		4,5BRPC	6,7BRPC	9,10BRPC	11,12BRPC
Native	339	33	29	37	47
108, 112	333	32	26	28	37
103, 108, 112	332	36	23	21	33

and 4.2, for the native and two mutants, respectively. The values for the two mutants are increased over that for the native protein since two Trps in the native protein were replaced by Leu in both mutants. In addition all proteins gave single bands on polyacrylamide gel electrophoresis.

Our previous studies on the topology of b_5 bound to lipid vesicles [1,9] have used two parameters to locate the fluorescent Trp within the depth of the membrane: the position of the emission maximum when protein is bound to 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles and the decrease in fluorescence intensity seen when the protein is bound to brominated lipids as compared to the intensity when it is bound to POPC. Studies with native b_5 are complicated by our recent report [1] which showed that Trp-109 is not the only fluorescent chromophore and accounts for only 2/3 of the total intensity. In spite of this complexity, it can be seen that the introduction of Leu causes a progressive blue shift in the emission maximum when the spectra of the lipid-bound proteins are examined (Table I). The fluorescence of the three proteins in different brominated lipids relative to their fluorescence in POPC is also shown in Table I. These data suggest there is a slight increase in the degree of quenching by bromine atoms deeper in the bilayer as Leu are introduced. In order to more accurately recover the changes in the Trp location in the two mutants a distribution analysis was performed as previously described [12]. In this analysis, the natural logarithms of the fluorescence intensities in the different BRPC's (normalized to the value in POPC vesicles) as a function of the distance of the bromines from the center of the membrane [13] is fitted to a Gaussian distribution. The two Gaussian distributions had the following parameters. For the (108, 112) mutant: maximum of the distribution (position of the Trp from the center of bilayer) 10.2 Å, full width at half-height (fwhh) 11.3 Å, area 9 relative units and for the (103, 108, 112) mutant: position 9.5 Å, fwhh 8.4 Å, area 8 relative units. These results indicate there is very little difference in the most probable depth of the Trp's in the two mutants (10.2 vs 9.5 Å) and the exposure of the Trp to the lipid phase (the areas under the two curves are within 10% of each other). The largest difference is observed in the width of the distribution: 11.3 Å for the (108, 112) mutant and 8.4 Å for the (103, 108, 112) mutant. This latter difference indicates that the (103, 108, 112) mutant has less conformational freedom to accept different positions in the membrane. The parameter fwhh for the Gaussian distribution actually contains contributions from the finite sizes of the Trp and the bromine as well as fluctuations in the depth positions of both of these.

In summary, both the fluorescence intensity and fluorescence quenching data indicate that these mutations are producing very little change in depth or exposure of the Trp and hence little change in conformation of the NPP. It should be emphasized that all these fluorescence data are compared only for the membrane-bound forms of the protein since the emission spectrum in aqueous solution is influenced by the state of aggregation of the b_5 (see below). Surprisingly, the loss of Asp-103 promotes only a slightly (less than 1 Å) deeper penetration of the Trp-containing sequence. It had previously been noted that residue 103 is highly conserved (all but chicken b_5 have either Glu or Asp) and it had been suggested that loss of the negatively charged amino acid would result in a much deeper penetration of the domain into the membrane [7].

Gel filtration of cytochrome b_5

The degree of self-association of the three proteins was evaluated by gel filtration. The elution properties of native b_5 and the two mutants are compared in Fig. 1. Native b_5, at low loading, gave a single included peak at 13.5 ml but at higher loadings, species with a smaller elution volume appeared with little increase in the concentration of the well included peak. With the highest concentration analyzed the elution profile consisted of a large peak eluted near the void volume followed by a plateau region. These results are essentially the same as previously reported [3] and are consistent with the presence of monomeric and octameric, and perhaps intermediate, species of the protein. When (108, 112) mutant b_5 was chromatographed at low loading, a single included peak with an elution volume similar to that seen with native b_5 (14 ml) was obtained. At higher loadings, although the peak became broader and eluted with a smaller elution vol-

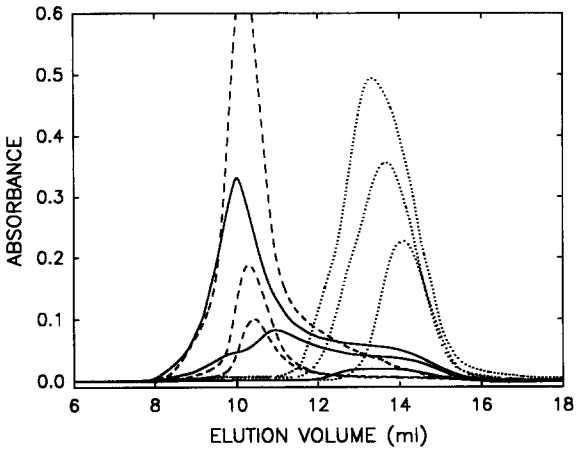

Fig. 1. Gel filtration of native and mutant cytochromes b_5. Gel filtration was performed as described in Materials and Methods. The following samples were analyzed in succession: native b_5 (solid lines) 6.8, 3.4 and 0.8 nmol; (108, 112) mutant b_5 (dotted lines) 6.8, 3.4 and 1.7 nmol; (103, 108, 112) mutant b_5 (dashed lines) 6.8, 3.4 and 1.7 nmol.

ume, there was no evidence of a peak corresponding to octameric b_5. Samples of (103, 108, 112) mutant b_5 at all concentrations gave only a single peak with an elution volume slightly larger than octameric native b_5. This indicates that the Stokes radius of the aggregate formed by (103, 108, 112) mutant b_5 is slightly smaller than the Stokes radius of the aggregate formed by the native b_5.

We have previously shown [3] that trace amounts of lipid in the b_5 sample will alter the elution position of native b_5 and, although no lipid was detected in any of these protein samples, it is conceivable that traces of lipid contaminating either of the mutant proteins could alter their elution properties. A control experiment was performed to eliminate this possibility. A mixture of (108, 112) and (103, 108, 112), which had been kept at 4°C for 24 h to allow for equilibration of any possible contaminating lipid, was subjected to gel filtration. This mixture gave two peaks with the same elution volumes as seen with low loading of the two mutants separately and indicates that the different elution profiles seen with the two mutants are not the result of lipid contamination of one of the samples.

Binding of cytochromes b_5 to lipids

The binding of native b_5 to lipid vesicles produces a large increase in fluorescence intensity and a binding isotherm can be generated by measuring the fluorescence enhancement as the lipid to protein ratio is increased [4]. Fig. 2 shows the fluorescence enhancement (normalized to the fluorescence value at saturating lipid) when the (103, 108, 112) mutant b_5, (108, 112) mutant b_5 and native b_5 are titrated with increasing concentrations of POPC small unilamellar vesicles. Binding curves of this type have previously been analyzed to obtain affinities and stoichiometries of binding [14]. When the binding data for the three b_5's were analyzed using the NONLIN program of Dr. Mike Johnson [15] the following values for dissociation constants, the number of lipids 'in the binding site' and [the confidence limits for 0.67 probability], were obtained: native, 56 nM [34, 68] and 66 'lipids' [63, 68] and for the (108, 112) mutant 79 nM [46, 122] and 158 'lipids' [151, 165]. No satisfactory fit could be obtained for the (103, 108, 112) mutant although the apparent K_d was of the order of μM (but see later). This affinity of the native protein for the vesicle is greater than that of the (108, 112) mutant for the vesicle and, at vesicle saturation there would be fewer mutant b_5's bound per vesicle than native b_5's bound per vesicle. The inability to analyze the binding curve seen with the (103, 108, 112) mutant (at an initial concentration of 0.7 μM) is due to the inadequacy of the model. With the (103, 108, 112) mutant, the binding experiments are complicated by the very low equilibrium concentration of monomer present in solution (from the gel filtration data it is less than 0.05 μM) such that at low lipid/protein ratios, most of the non-bound b_5 is in an aggregated form rather than being present as monomer. To examine this possibility, several of the samples were subjected to a ten-fold dilution and, as shown in Fig. 2, the curvature and the slope of the initial portion of the curve increased somewhat. In addition, it was found that the overall enhancement in fluorescence at high lipid/protein ratios (fluorescence of lipid bound b_5/

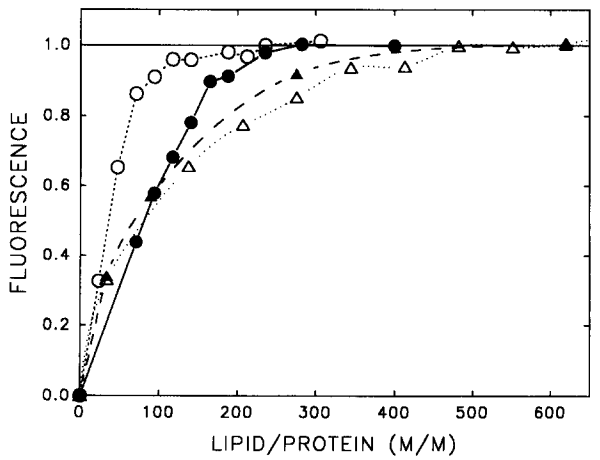

Fig. 2. Normalized increase in tryptophanyl fluorescence upon binding to lipid vesicles. The samples were prepared and fluorescence was measured as described in Materials and Methods. The fluorescence data were normalized to give the overall increase in fluorescence at high lipid/protein ratios to be equal to 1.0. The actual overall enhancements in fluorescence for each of the samples at high lipid/protein ratio (fluorescence of lipid bound b_5/fluorescence of initial free b_5) were: native b_5, 1.8-fold (open circles); (108, 112) mutant b_5, 2.3-fold (closed circles); (103, 108, 112) mutant at 0.7 μM, 2.0-fold (open triangles); and (103, 108, 112) mutant b_5 at 0.07 μM, 2.3-fold (closed triangles).

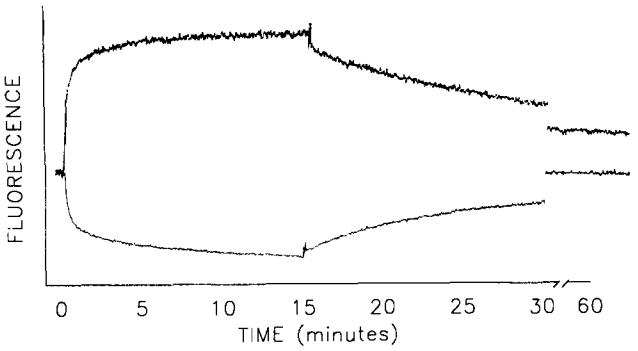

Fig. 3. Time-course of the changes in fluorescence upon adding lipid vesicles to native b_5. The upper tracing is for addition first of POPC vesicles then BRPC vesicles, the lower tracing is for addition of BRPC then POPC.

fluorescence of initial free b_5) was 2.0-fold for the more concentrated solution of (103, 108, 112) mutant b_5 and 2.3-fold for the diluted sample. This difference in enhancement is likely due to a lower quantum yield of free monomeric protein compared to the aggregated protein and suggests there was more monomeric protein present in the diluted protein sample initially. However, the total concentration of the (103, 108, 112) mutant b_5 at 0.07 μM is still higher than the equilibrium concentration of monomer.

Exchange of cytochromes b_5 between lipid vesicles

Cytochrome b_5, in common with other amphiphiles, has the property of exchanging between lipid vesicles [5]. The rate of this process can be monitored by following the change in fluorescence as the protein moves from a normal lipid ('enhancing') vesicle to a brominated lipid ('quenching') vesicle, or vice versa. In addition, the initial rate of fluorescence change when the lipid is first added to the aqueous protein gives an estimation of the rate of binding. The rate of enhancement of fluorescence of native b_5 was multiphasic [4] and a half-time of 30 s was obtained for the overall enhancement process. The half-time decreased to approximately 3 s with (108, 112) and increased to 500 s with (103, 108, 112). The half-times for exchange of native b_5, (108, 112) and (103, 108, 112) were: 15 min, 20 s and 60 min, respectively. The time-course for such a binding and exchange experiment is demonstrated for native b_5 in Fig. 3.

Discussion

The ability to express b_5 in micro-organisms has enabled mutants to be generated which have properties which are advantageous for biophysical studies [6] as well as allowing the correlation between amino acid sequence and enzymic properties to be probed [2,8]. The highly conserved amino acid sequence seen in the NPP of b_5 from different species implies that this sequence is optimal for the function of the protein. Two mutants have been initially investigated by us, one had two Trp residues replaced by Leu while the second had, in addition, an Asp replaced by Leu. Leucine was chosen as the substitute amino acid to ensure the mutants had high membrane affinity but during the fluorescence experiments it became apparent that these three amphipathic proteins had very different degrees of self association. The relative hydrophobicities of Leu and Trp have been extensively debated and, in fact, on some scales their hydrophobicities are reversed [16]. In studies on the membrane partitioning of a series of tripeptides it was found that Trp was more 'hydrophobic' than Leu, although it was noted that these peptides reside mainly in the interface region [17]. Recent work from this latter group indicated that, because of the finite solubility of water in the cyclohexane used for partitioning experiments, the indole NH of the Trp is considerably less polar than previously thought [18]. Because of these practical and theoretical conflicts we considered it would be valuable to use these two mutants of b_5 to compare directly the relative hydrophobicities of these two amino acid residues in a membrane protein.

From our previous studies with native b_5 we have available three assays which can be used to estimate hydrophobicity: self-association, membrane-partitioning, and rates of membrane exchange. The gel filtration behavior, assuming that no absorption artifacts occur, gives a qualitative view of the strength of self-association but can also be used to give approximate values of the different free energies of association. If it is assumed that all the proteins do self-associate into an octameric micellar structure at sufficiently high concentrations and the maximum values of monomeric species seen eluting from the column are the approximate values of the critical micelle concentrations (cmc) then these are: 0.5 μM, 2.0 μM and less than 0.05 μM, for the native b_5, the (108, 112) and the (103, 108, 112) mutant b_5 respectively. Following the analysis of Tanford [19] for detergent micelles, the ΔGs for transfer from water to the micelle ($RT\ln$ (cmc/55.6), to allow for the cratic contribution) are approximately: -10.8, -9.9 and -12.1 kcal mol^{-1}, respectively. These can be compared to the changes calculated to be produced by the amino acid substitutions made. If the 'consensus' scale of Eisenberg [16] is used, the values for Leu, Trp and Asp are 0.53, 0.37 and -0.72 kcal mol^{-1}, respectively. Hence the ΔG for transfer from water to a micelle for the (108, 112) mutant b_5 should be -11.1 kcal mol^{-1} and for the (103, 108, 112) mutant b_5 it should be -12.4 kcal mol^{-1}, based on our experimentally determined value for native b_5. Here the value for the (108, 112) mutant b_5 is very different from that predicted by the gel filtration data. If the 'atom based' scale of Eisenberg and McLachlan [20] is used, where

Leu, Trp and Asp are 1.9, 2.6 and −1.2 kcal mol^{-1}, respectively the ΔG value for the (108, 112) mutant is −9.4 kcal mol^{-1} and for the (103, 108, 112) mutant is −12.5 kcal mol^{-1}. Here the values for all three of the proteins rank in the same order as the gel filtration data.

The binding experiments with the three proteins gave calculated dissociation constants which should be interpreted with caution. Only in the case of the (108, 112) mutant are we sure that the protein species at the start of the titration was the monomeric species and we can therefore analyze the data by a two-state model [14] to give a K_d of 79 nM. The native protein had tighter binding ($K_d = 56$ nM) but the initial protein concentration of 1.7 μM is higher than the estimated cmc (0.5 μM) and so the measured K_d may be somewhat high due to the presence of octameric b_5, the true K_d for the native protein is likely less than 56 nM. This problem is exaggerated with the (103, 108, 112) mutant, the K_d of which appears in the micromolar range due to the very low cmc (0.05 μM) of this mutant. Although it is possible, in theory, to allow for the presence of octameric protein it is likely that the system is further complicated by the presence of mixed micelles of lipid and protein and so no further analysis of these binding curves was attempted.

Although cytochrome b_5 is an integral membrane protein it, in common with other amphiphiles, has the property of exchanging between lipid vesicles [5]. We showed that this process can be conveniently monitored by following the fluorescence changes as the protein moves from a normal lipid ('enhancing') vesicle to a brominated lipid ('quenching') vesicle, or vice versa. In addition, the initial rate of fluorescence change when the lipid is first added to the aqueous protein gives an estimation of the rate of binding. If both of these rates can be determined, their ratio is a measure of the membrane affinity. Our previous studies showed that binding of monomeric protein was very rapid ($\tau_{1/2} < 500$ μs) whereas exchange between vesicles occurred with a half-time of several minutes [4,5]. The kinetics of fluorescence enhancement are complicated, as noted previously, by the presence of both monomer and octamer in solution. The very fast, 'instantaneous', increase which is seen is due to monomer binding, the slower rate to dissociation of octamer, followed by instantaneous binding of the monomer formed [4]. The decreased half-time seen with (108, 112) is certainly a reflection of the preponderance of monomeric protein but no estimate of the actual rate of monomer binding can be made. Similarly, the slow rate of binding seen with (103, 108, 112) is due to the very low level of monomeric protein and a very slow rate of dissociation of octamer. It is quite likely, indeed probable, that the rate of binding of monomeric (103, 108, 112) is just as fast as native b_5. The rates of exchange are more accessible. The half-times for native b_5, (108, 112) and (103, 108, 112) were: 15 min, 20 s and 60 min, respectively. These rates of exchange rank in the same order as the ΔG values of 'micellization'. These kinetic experiments, with the reasonable assumption that the 'on rates' for the monomeric forms are equal for all b_5's, as with most amphiphiles [21] then give a measure of the membrane affinity (K_d = OFF rate/ON rate). The relative K_d's obtained from these rates of exchange, together with the absolute K_d (79 nM, 1.42×10^{-9} mole fraction units) obtained above for the (108, 112) mutant by the lipid titration experiment, enable the remaining K_d's and the ΔG values for binding of the proteins to lipid vesicles to be estimated, again correcting for the cratic contribution: native 1.8 nM, −14.0 kcal mol^{-1}; (108, 112) mutant 79 nM, −11.8 kcal mol^{-1}; (103, 108, 112) mutant 0.45 nM, −14.8 kcal mol^{-1}. The ΔG values of self-association from gel filtration were −10.8, −9.9 and −12.1 kcal mol^{-1}, respectively.

All these data consistently indicate that the strengths of self-association and membrane-association of the three proteins rank in the order: (103, 108, 112) mutant > native > (108, 112) mutant. The consequences of this to the structures of the complexes are also internally consistent. The (108, 112) mutant has a lower affinity than the native b_5 for vesicles and this results in a lower number of proteins bound per vesicle at saturation. In terms of 'opposing forces' [19] this lower number could result from the repulsion between the 'constant' charged polar domains overwhelming the weaker binding of the (108, 112) membrane-binding domain. Similarly, the effect of the greater hydrophobicity of the (103, 108, 112) mutant b_5 is seen in the gel filtration behavior of the aggregated forms of native versus (103, 108, 112) mutant b_5. The elution volume of the aggregate seen with this mutant is greater than that seen with the native protein, which implies the mutant aggregate has a smaller Stokes radius. We had previously commented [22] that the Stokes radius of the native aggregate was much larger than predicted for a simple globular protein of equal mass and suggested that the octameric native b_5 had quite an open structure, because the repulsion between the charged polar domains dominated over the hydrophobicity of the membrane-binding domains. The smaller size of the aggregate of (103, 108, 112) would be in keeping with the greater hydrophobicity being better able to overcome the repulsion between the same polar domains.

These data indicate that small changes in amino acid sequence cause dramatic changes in the hydrophobicity of this membrane-binding domain. There is evidence that the changes in hydrophobicity are simply the result of the amino acid substitutions rather than gross conformational alterations in the membrane-binding domain. The changes seen in the fluorescence

emission spectra of the three proteins are consistent with the depths of the Trp as estimated by bromolipid quenching and the relative overall quenching by the bromolipids indicates the 'exposure' of the Trp to these quenchers is unaltered. FTIR analysis also suggests the isolated nonpolar domains of the mutants are still predominantly α helical [6,23,24]. The observed self-association and relative membrane-affinities agree, probably within the limits of our data, with the 'atom based' scale of Eisenberg and McLachlan [20]. It is of interest that all native b_5's have three conserved Trp in the membrane-binding domain and, in this very limited series of three proteins investigated, the native b_5 with three Trp has an intermediate hydrophobicity. It may be that the very properties which make the hydrophobicity of Trp difficult to assign are those required for the correct functioning of this protein.

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References

1 Ladokhin, A.S., Wang, L., Steggles, A.W. and Holloway, P.W. (1991) Biochemistry 30, 10200–10206.
2 Vergères, G. and Waskell, L. (1992) J. Biol. Chem. 267, 12583–12591.
3 Calabro, M.A., Katz, J.T. and Holloway, P.W. (1976) J. Biol. Chem. 251, 2113–2118.
4 Leto, T.L. and Holloway, P.W. (1979) J. Biol. Chem. 254, 5015–5019.
5 Leto, T.L., Roseman, M.A. and Holloway, P.W. (1980) Biochemistry 19, 1911–1916.
6 Ladokhin, A.S., Tretyachenko-Ladokhina, V.G., Holloway, P.W., Wang, L. and Steggles, A.W. (1992) Biophys. J. 62, 79–81.
7 Ozols, J. (1989) Biochim. Biophys. Acta 997, 121–130.
8 Ladokhina-Tretyachenko, V.G., Ladokhin, A.S., Wang, L., Steggles, A.W. and Holloway, P.W. (1992) FASEB J. 6, A85.
9 Markello, T., Zlotnick, A., Everett, J., Tennyson, J. and Holloway, P.W. (1985) Biochemistry 24, 2895–2901.
10 Dariush, N., Fisher, C.W. and Steggles, A.W. (1988) Protein Sequences Data Anal. 1, 351–353.
11 Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
12 Ladokhin, A.S., (1993) Biophys. J. 62, A290.
13 McIntosh, T.J. and Holloway, P.W. (1987) Biochemistry 26, 1783–1788.
14 Hille, J.D.R., de Kelder, G.M. D-O., Sauve, P., de Haas, G.H. and Egmond, M.R. (1981) Biochemistry 20, 4068–4073.
15 Johnson, M.L. and Frasier, S.G. (1985) Methods Enzymol. 117, 301–342.
16 Eisenberg, D. (1984) Annu. Rev. Biochem. 53, 595–623.
17 Jacobs, R.E. and White, S.H. (1989) Biochemistry 28, 3421–3437.
18 Wimley, W.C. and White, S.H. (1992) Biochemistry 31, 12813–12818.
19 Tanford, C. (1980) The Hydrophobic Effect (2nd Edn.), Wiley, New York, p. 62.
20 Eisenberg, D. and McLachlan, A.D. (1986) Nature (London) 319, 199–203.
21 Cevc, G. and Marsh, D. (1987) Phospholipid Bilayers: Physical Principles and Models, Wiley, New York, pp. 51–55.
22 Holloway, P.W. and Katz, J.T. (1975) J. Biol. Chem. 250, 9002–9007.
23 Holloway, P.W. and Mantsch, H.H. (1989) Biochemistry 28, 931–935.
24 Holloway, P.W. and Buchheit, C. (1990) Biochemistry 29, 9631–9637.