Saturable binding of various inhaled anesthetics to serum albumin has been shown with a variety of approaches. In order to determine the location of halothane binding sites in serum albumin, both human and bovine serum albumins (HSA and BSA) were photolabeled with [14C]halothane, and subjected to proteolysis and microsequencing. BSA was found to have a higher affinity for halothane than HSA, and it contained two specifically labeled sites. One site was characterized by diffuse labeling from Trp212-Leu217, and the other by a more discrete and higher affinity labeling at Trp134-Gly135. HSA contained only a single labeled site, and although lower affinity, was determined to be analogous to BSA Trp212. The position 130–140 region of HSA, having a leucine instead of tryptophan at position 134, was not labeled. These results demonstrate specific and discrete binding of an inhaled anesthetic to a mammalian-soluble protein, and further suggest the importance of aromatic residues as one feature of inhaled anesthetic binding sites.

Volatile inhalational anesthetics can bind to and alter the function of a variety of proteins, both soluble and membrane-bound (1). Determination of the characteristics of these binding sites in proteins should allow prediction of the interactive forces producing binding (immobilization) and also hint at the structural or dynamical consequences to the target. Furthermore, the demonstration of a binding motif common to many protein targets will simplify the search for anesthetic binding domains in other systems. Unfortunately, the character of anesthetic binding sites in protein has been difficult to determine directly, and few examples are available.

While consensus places important actions of anesthetics at membrane protein, the presence of lipid and multiple apparently specific binding sites (2) renders these complex models presently unsuitable for a focused search of binding site features. Certain soluble proteins, on the other hand, appear to have a limited number of discrete binding sites for inhalational anesthetics, making them reasonable initial candidates for characterizing anesthetic binding domains. Accordingly, several approaches have identified specific, saturable binding of halothane in bovine serum albumin (3–6) and have also demonstrated anesthetic-induced alterations in the structure or carrier function (7–12) of this protein. In order to localize the actual binding sites in this soluble mammalian protein, I have used direct photoaffinity labeling with [14C]halothane (5, 13) to covalently link this commonly used inhalational anesthetic to albumin sites normally occupied under equilibrium binding conditions.

EXPERIMENTAL PROCEDURES

Materials—Fatty acid-free bovine and human serum albumins were used as purchased from Sigma. Endoprotease Glu-C and Asp-N, and other listed chemicals were also obtained from Sigma. [14C]Halothane ([1-14C]bromo-2-chloro-1,1,1-trifluoroethane; 6.6 mCi/mmol) was purchased from DuPont NEN as the neat compound, which was dissolved in buffer as a stock 6 mM solution and stored at –80°C. Electrophoresis reagents and supplies were purchased from Bio-Rad.

Photoaffinity Labeling—Albumin solutions (generally 15 μM) in deoxygenated (argon-bubbled) 150 mM NaCl, containing various concentrations (0.05–0.4 mM) of [14C]halothane exposed to 254 nm light (Oriel Hg pencil calibration lamp at 7 mm) for 60–100 s in 5-mm path-length quartz cuvettes (2 ml) at 20–22°C. The final addition completely filled the cuvette, which was then sealed with a Teflon stopper, so there was no gas space during equilibration and photolysis. This permitted predictable anesthetic concentrations to be achieved by dilution, occasionally verified by extraction of aliquots into hexane and subsequent gas chromatographic measurement. About 1 min was allowed for equilibration prior to photolysis, and the cuvette contents were continuously mixed with microstrip bars. No attempt was made to label albumin to completion with halothane by prolonged UV exposures, because of the potential for cross-linking and unpredictable alterations of protein structure with 254 nm light. Therefore, the determined binding stoichiometry is an underestimate of the true concentration of binding sites. After UV exposure, the cuvette contents were either precipitated with 5% trichloroacetic acid, filtered through GF/B filters, or washed by repeated centrifugation in Centricon 10-kDa cutoff filters (Amicon, Beverly MA).

Competition Experiments—To determine the Ks for halothane in human and bovine serum albumin, photoaffinity labeling was performed as above ([14C]halothane) ~ 40–50 μM with increasing concentrations of unlabeled halothane (from a 12 mM stock solution) included in the cuvette. Cuvette contents were precipitated and filtered; labeling was determined by liquid scintillation counting of the GF/B filters. Data were fitted to sigmoid curves using nonlinear least squares regression analysis to compute the IC50, Ks, and Hill coefficients. In addition, the influence on halothane binding of prior fatty acid (oleic acid) loading (10:1 stoichiometry) or pH < 3.5 conditions (which unfolds serum albumin to the "E" form (14)) was determined. Fatty acids were loaded on albumin as described previously (3, 4). For each of these conditions, an aliquot was resuspended in 70% formic acid for CNBr digestion (see below), and then in sample buffer for electrophoresis and autoradiography.

Digestions—Aliquots of washed and labeled albumin (photolabeled at [14C]halothane) ~ 0.5 mM) were lyophilized and resuspended in 70% formic acid, and then digested with a 1000-fold molar excess (over Met residues) of CNBr for 24 h under argon in the dark at 20°C. After dilution and lyophilization, the samples were resuspended in sample buffer (with mercaptoethanol), heated and run on SDS-polyacrylamide gels.
Acid over albumin sites reduced label incorporation in BSA by 40–50 mM halothane reduced labeling of BSA by 84%.

PVDF, polyvinylidene difluoride; HPLC, high pressure liquid chromatography; BSA, bovine serum albumin; HSA, human serum albumin.

Tricine gels. Stained bands corresponding to the predicted complete digestion fragments were excised from the gel, dissolved in 30% H2O2 at 65 °C for 12 h, and label content determined with liquid scintillation counting (with correction for peroxide chemiluminescence). Specifically labeled albumin fragments were identified by comparing CNBr fragment counts in protein that was labeled in the presence or absence of 5 mM unlabeled halothane, and then purified out of larger scale labeling experiments using continuous elution gel electrophoresis in a Bio-Rad Prep-Cell. Further treatment of these purified CNBr fragments is described under “Results.”

Sequencing—Digests or purified fragments were dissolved in sample buffer and run on pre-electrophoresed SDS-PAGE Tricine gels with 0.1 mM thioglycolic acid in the running buffer. The gels were briefly washed with distilled water and electrophoretically blotted to PVDF membranes, which were then stained, dried, and the appropriate bands sequenced on an Applied Biosystems model 473A sequencer. The label content of each cycle was determined by prolonged scintillation counting of the saved HPLC fractions.

RESULTS

Competition Experiments—Sixty-second UV exposures of BSA in the presence of 40–50 μM [14C]halothane-labeled bovine serum albumin (BSA) with a stoichiometry of 1:2 (label:albumin), human serum albumin (HSA), on the other hand, under identical conditions, labeled at a significantly lower ratio of about 1:15. Fig. 1 shows that unlabeled halothane reduced label incorporation with a Ki of 0.18 ± 0.02 for BSA and 0.72 ± 0.12 for HSA, both with Hill coefficients of about −1. Five mM unlabeled halothane reduced labeling of BSA by 84 ± 3% and HSA by 73 ± 2% while acid conditions, producing the extended “E” conformation, was reduced label incorporation in BSA by 72 ± 2% and HSA by 55 ± 8%, and the incorporation of excess oleic acid over albumin sites reduced label incorporation in BSA by 57 ± 8% and HSA by 26 ± 3% (Table I). These results show that the labeling of albumin by halothane is largely specific, that BSA has a larger specific binding component than HSA, that the native conformation is required for specific binding, and that halothane sites interact with the known fatty acid sites on these proteins.

BSA Digestions—CNBr digestion of BSA produced 5 fragments, consistent with the known number (four) and distribution of methionine residues, and the resulting electrophoresis is shown in Fig. 2. Of these five fragments, only two appear to contain significant label: a 29-kDa fragment (CNBr-29) and a fragment at about 11 kDa (CNBr-11). Together, these two fragments accounted for about 80% of the label in the entire 66-kDa protein, and the labeling of both was substantially reduced by 5 mM halothane, fatty acid loading, and acid conditions as given in Table I. However, significant differences existed between the labeling of these two fragments. In general, CNBr-11 contained twice as much label as CNBr-29 and was reduced by a greater extent by 5 mM unlabeled halothane, fatty acids, and low pH. Since this may be interpreted as reflecting higher affinity, the Ki for each CNBr fragment was determined in samples of labeled proteins from competition experiments. Consistent with this premise, Fig. 3 shows that a significantly lower apparent Ki was obtained for CNBr-11 than for CNBr-29.

Previous tryptophan fluorescence quenching studies (6) suggested immobilization of the halothane near the two tryptophan residues in BSA, both of which are contained in the specifically labeled CNBr fragments. Further cleavage of CNBr-29 with endoprotease Glu-C in 50 mM ammonium carbonate buffer (pH 8) produced a 5-kDa fragment containing 69% of the initial CNBr-29 label. Microsequencing of this 5-kDa piece showed that it was the N terminus of CNBr-29 (REKVL...). Therefore, in order to locate the label on this fragment, approximately 30 μg of the entire 29-kDa fragment containing about 1600 cpm, was loaded on PVDF membranes and sequenced. After confirmation of the sequence with the identification of 3 amino acids (REK...), 38 subsequent cycles were performed (without HPLC identification, to minimize loss.
of label) and cpm determined with liquid scintillation counting. Fig. 4 demonstrates a labeling peak that stretches from Trp212 to Leu217.

It was not initially clear which of three similar sized fragments resulting from CNBr digestion of BSA that the labeled CNBr-11 represented, and the three fragments were not always well resolved from each other (see Fig. 2). Possibilities included: Asp1–Met86, Ala87–Met183, or Pro445–Met546, but we suspected the fragment with Trp134. Because only the W134-containing fragment has a DP bond at 118–119, an elution fraction from preparative PAGE with all three fragments was incubated in 70% formic acid for 48 h at 37°C to cleave this acid-sensitive bond. After this mild hydrolysis, electrophoresis on Tricine gels showed that a 7-kDa fragment containing C124, confirmed the expected sequence, but failed to yield any significant labeling in the same region as BSA (see Discussion). CNBr-19 was purified with preparative PAGE and further digested with endoprotease Asp-N in 10 mM Tris-HCl. A 5-kDa fragment containing 60% of the loaded label was found to start at Asp137, being long enough to just span the region containing the conserved Trp at position 214. Because of the relatively low labeling of this fragment, and the high likelihood of similarity to BSA in this conserved site, sequencing was not done.

**DISCUSSION**

The volatility, low affinity, and rapid kinetics of the inhaled anesthetic molecules have precluded application of conventional binding studies in biologic tissues and macromolecules, and this has undoubtedly contributed to the slow progress in understanding mechanisms of action. Photoaffinity labeling converts the rapid equilibrium kinetics to irreversible covalent bonds, permitting more conventional biochemical approaches to define binding parameters and localize sites. The precise photochemistry involved with direct halothane photo-
Direct photoaffinity labeling with halothane has demonstrated the existence of saturable binding in a number of model systems, including biologic membranes (2, 13) and soluble proteins (4). In the latter case, saturable binding of inhalational anesthetics to BSA has also been demonstrated with gas chromatography partition analysis, \(^{13}F\) NMR spectroscopy (3, 5), and intrinsic tryptophan fluorescence quenching (6). Dissociation constants for the BSA/halothane interaction, and the effect of low pH, fatty acid, and excess unlabeled compound have been remarkably similar across these approaches. Thus far, however, tryptophan fluorescence quenching is the only approach that begins to localize the anesthetic within the albumin molecule to the vicinity of the two known tryptophan residues. Inhibition of anesthetic binding by fatty acid (3, 5) implicates the fatty acid binding domains within this protein as anesthetic binding sites, but this is ambiguous because of the well known allosteric nature of albumin, and the fact that multiple such domains exist, all of which are not well localized. Anesthetic alteration of the chirality of bilirubin binding (11) suggests that the IIA binding domain (14) may bind anesthetics, but this is also ambiguous because of the potential for allosterism. Therefore, localization of the actual binding sites for an inhalational anesthetic using photoaffinity labeling was a rational next goal, so that interactions within this site can be explored, characterized and possibly optimized with new drugs.

In BSA and HSA, a binding site for halothane is found in the immediate vicinity of the conserved tryptophan (212 for BSA, 214 for HSA). This site is within domain IIA (14), and has been characterized as binding small charged but hydrophobic molecules like bilirubin, warfarin, and triiodobenzoic acid (17). Consistent with localization in this site are data that show an alteration of bilirubin binding by inhalational anesthetics (11) and an ability of halothane to alter warfarin binding (9). Domain IIA does not bind fatty acids, suggesting that fatty acid inhibition of halothane binding is an allosteric effect. Therefore, these photolabeling results are consistent with the previously reported tryptophan fluorescence quenching data, and point toward aromatic side chains as a potentially important structural feature of halothane binding sites.

The importance of the tryptophan indole in producing halothane binding is reinforced by the second site in BSA: Trp134. In BSA and HSA, a binding site for halothane is found in the immediate vicinity of the conserved tryptophan (212 for BSA, 214 for HSA). This site is within domain IIA (14), and has been characterized as binding small charged but hydrophobic molecules like bilirubin, warfarin, and triiodobenzoic acid (17). Consistent with localization in this site are data that show an alteration of bilirubin binding by inhalational anesthetics (11) and an ability of halothane to alter warfarin binding (9). Domain IIA does not bind fatty acids, suggesting that fatty acid inhibition of halothane binding is an allosteric effect. Therefore, these photolabeling results are consistent with the previously reported tryptophan fluorescence quenching data, and point toward aromatic side chains as a potentially important structural feature of halothane binding sites.

The importance of the tryptophan indole in producing halothane binding is reinforced by the second site in BSA: Trp134. This site had a higher affinity for halothane than BSA Trp312, but did not produce binding at all in the absence of the tryptophan, as in the case of HSA. However, other differences exist in
this region of albumin. Considering only the primary structure, there is approximately 60% homology for the 120–150 stretch, but it is difficult to reconcile this with the actual site without a published BSA crystal structure. If the remainder of this site is similar to HSA, this natural-directed site mutagenesis experiment implies that indole provides an important interaction that immobilizes halothane in this more water-exposed site on BSA. This interaction is probably electrostatic, since the leucine in this position of HSA is more hydrophobic than tryptophan (18), but lacks the potential electrostatic interactions of the aromatic ring hydrogens or π-electrons. Since covalent binding is likely to occur at the 2-carbon end of halothane, this logic would suggest that the C-C axis of halothane is oriented perpendicular to the indole ring plane, with the relatively electronegative 2-carbon (hydrogen?) interacting with the π-electrons. Such π-cation interactions are now suspected to be of general importance in receptor/ligand interactions (19), and may help to reconcile previous studies, which suggest that functional anesthetic sites have both hydrophobic and polar character (20, 21). This assumes that photochemical binding occurs in the reversible equilibrium-bound orientation of the halothane molecule. This is consistent with halothane’s quenching of BSA tryptophan fluorescence, thought to result from proximity of the bromine atom on the 2-carbon to the indole moiety (6), and also chlorotrifluoroethyl radical half-life estimates (5), which are more than 2 orders of magnitude faster than halothane dissociation rates (3). However, should the chlorotrifluoroethyl radical be long-lived relative to the dissociation rate, different conclusions might apply. For example, the polarity of the radical would be expected to be opposite to that of the parent molecule, and therefore may reverse its orientation with respect to the indole π-electrons. Alternatively, the radical may alter its orientation more subtly to interact with the electropositive hydrogens of the indole ring, specifically the N-H because of the higher partial charge on this proton. However, since the data indicate that binding occurs between the radical 2-carbon and the tryptophan, the latter seems more likely. Regardless, the simplest interpretation of these data is that interactions between the relatively positively charged end of halothane and the indole π-electrons explain immobilization in the Trp134 site. This is less clear for the domain IIA site, largely because of the greater number of amino acids labeled. Presumably, this wider distribution reflects weak halothane binding, suggesting a larger volume site and/or a less optimal distribution of interactive side chains.

That hydrophobic sites containing aromatic residues are favored binding sites for inhalational anesthetics is not entirely new. Low resolution x-ray crystallography of adenylyl kinase has localized halothane to a discrete interhelical niche lined with hydrophobic residues, as well as more polar aromatics like tyrosine (22). Also shown with x-ray diffraction, the active site of the enzyme haloalkane dehalogenase, which binds and catalyzes the dechlorination of dichloroethane (23), contains two tryptophans and two phenyalanines, whose protons are thought to interact electrostatically with the halogens of the substrate. Finally, the much less potent anesthetic gases, such as xenon and nitrous oxide, have also been reported to localize close to aromatic residues in a variety of proteins like myoglobin and albumin, principally through van der Waals interactions (24–27). Thus, binding sites for these small molecules may have common features, and in fact be quite widely distributed, consistent with the growing view of a multisite nature of their action.

The labeling of these tryptophan sites is unlikely to result from simple photochemical selectivity for the following reasons. First, intrinsic tryptophan fluorescence was quenched by halothane with similar dissociation constants as found with photoaffinity labeling (6), implying equilibrium binding in the vicinity of these residues. Second, other proteins that contain tryptophan in hydrophobic environments, like myoglobin, have no saturable binding sites for halothane based on photoaffinity labeling1 tryptophan fluorescence quenching (6), or x-ray diffraction experiments (24), presumably due to lower cavity volumes or access. Third, other aliphatic and even charged residues were labeled by halothane in this study (in domain IIA especially). Fourth, this study found significant quantitative differences in binding to the 3 halothane accessible, W-containing sites in BSA and HSA. Finally, photos labeling experiments with poly-(L-lysine), resulted in significant saturable halothane binding only when the homopolymer was in a conformation that created hydrophobic environments (pH > 11) (16), and such labeling was increased by only 50% in random polymers of lysinetransferase (4:1), defining the upper limit of aromatic selectivity. Taken together, these data demonstrate the overriding importance of conformation and equilibrium binding as opposed to photochemical selectivity in direct halothane photoaffinity labeling.

The greater number of inhalational anesthetic sites in BSA as compared to HSA may be reflected in a functional difference between these two proteins, but little data that directly compares these two proteins is available, especially in the presence of anesthetics. It is known, for example, that BSA binds fatty acids more tightly than HSA (14), consistent with the lower ability of fatty acids to displace halothane from HSA than from BSA (this study). Also, bilirubin is bound in opposite chirality by BSA and HSA (14), indicating that steric features in the domain IIA cavity must differ, possibly reflected by the differences in apparent halothane affinity of this same site in BSA versus HSA. Finally, despite many similarities, there is only 78% sequence homology between the two albums, so it is not unreasonable to expect that a difference in anesthetic binding behavior, and probably also the structural and functional consequences of such binding, to exist.

As stated in the Introduction, the ultimate sites of inhalational anesthetic action are thought to be the membrane proteins, and probably not soluble proteins. Thus far, however, discrete protein binding sites for these interesting compounds have only been described in soluble proteins, such as adenylyl kinase (22), serum albumin (this study, and Refs. 3–6 and 27), myoglobin (24–26), and possibly firefly luciferase (1). Binding studies in the membrane proteins are more problematic, in part because such studies with low affinity gases is technically challenging, but also because of an unsubstantiated concern that “nonspecific” partitioning in the membrane lipid will overwhelm specific protein binding at functionally relevant concentrations. It might be difficult, for example, to distinguish specific binding to transmembrane segments of such proteins from simple partitioning into the bordering bulk lipid. To further complicate matters, it is possible that the lipid-protein interface is the favored binding site for inhaled anesthetics (29–31). This is, of course, an entirely plausible site of action as well, due to the role of these generally conserved transmembrane sequences in the functioning of ion channels, receptors, and transporters. Reinforcing this important functional role are studies which show that synthetic peptides consisting only of transmembrane helical bundles can gate and selectively conduct ions (32). However, anesthetic effects at the lipid/protein interface have been difficult to demonstrate, but it is clear that alteration of the lipid composition can alter the influence of anesthetics on channel function (33). Specific mutations in the

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1. R. G. Eckenhoff, unpublished studies.
pore-forming transmembrane sequence of the nAChR alter its sensitivity to general anesthetics (34), which, while suggesting the importance of these regions for function, do not necessarily define anesthetic binding sites. We have recently reported evidence of multiple specific halothane binding sites in nAChR from *Torpedo nobiliana* using direct photoaffinity labeling, greater than 90% of which are in digestion fragments containing the transmembrane regions (2). This is in contrast to the few discrete sites found in serum albumin and other soluble proteins and suggests that fundamental differences in the binding of inhalational anesthetics to soluble versus membrane proteins exist, neither of which can as yet be disregarded as potential sites of action.

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REFERENCES

1. Franks, N. P., and Lieb, W. R. (1994) *Nature* **310**, 607–614
2. Eckenhoff, R. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.*, **93**, 2807–2810
3. Dubois, B. W., and Evers, A. S. (1992) *Biochemistry* **31**, 7069–7076
4. Dubois, B. W., Cherian, S., and Evers, A. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6478–6482
5. Eckenhoff, R. G., and Shuman, H. (1993) *Anesthesiology* **79**, 96–106
6. Johansson, J. S., Eckenhoff, R. G., and Dutton, P. L. (1995) *Anesthesiology* **83**, 316–324
7. Balasubramanian, D., and Wetlaufer, D. B. (1966) *Proc. Natl. Acad. Sci. U. S. A.* **55**, 762–765
8. Dale, O. (1986) *Biochem. Pharmacol.* **35**, 557–561
9. Calvo, R., Aguilera, L., Suarez, S., and Rodriguez-Sasian, J. M. (1989) Acta Anaesthesiol. Scand. **33**, 575–577
10. Buch, H. P., Altmayer, P., and Buch, U. (1990) Acta Anaesthesiol. Scand. **34**, 35–40
11. McDonagh, A. F., Pu, Y. M., and Lightner, D. A. (1992) *Experientia* (Basel) **48**, 246–248
12. Suarez, E., Aguilera, L., Calvo, R., Rodriguez-Sasian, J. M., and Martinez-Jorda, R. (1991) *Methods Find. Exp. Clin. Pharmacol.* **13**, 693–696
13. El-Maghrabi, E. A., Eckenhoff, R. G., and Shuman, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4329–4332
14. Peters, T., jr. (1985) *Adv. Protein Chem.* **37**, 161–248
15. Ruoho, A. E., Rashidbaigi, A., and Roeder, P. E. (1984) in *Membranes, Detergents and Receptor Solubilization*, pp. 119–160, Alan R. Liss, Inc., New York
16. Johansson, J. S., and Eckenhoff, R. G. (1996) Biochim. Biophys. Acta, in press
17. Carter, D. C., and Ho, J. X. (1994) *Adv. Protein Chem.* **45**, 153–203
18. Cornette, J. L., Casey, C. B., Margalit, H., Spouge, J. L., Berzofsky, J. A., and DeLisi, C. (1987) *J. Mol. Biol.* **195**, 659–678
19. Dougherty, D. A. (1996) *Science* **271**, 163–168
20. Hansch, C., Vitoria, A., Silipo, C., and Jow, P. Y. C. (1975) *J. Med. Chem.* **18**, 546–548
21. Franks, N. P., and Lieb, W. R. (1978) *Nature* **274**, 339–342
22. Sachsenheimer, W., Pai, E. F., Schulz, G. E., and Schirmer, R. H. (1977) *FEBS Lett.* **79**, 310–312
23. Verscheuren, K. H. G., Seljee, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) *Nature* **363**, 693–698
24. Schoenborn, B. P. (1967) *Nature* **214**, 1120–1122
25. Schoenborn, B. P., Watson, H. C., and Kondrew, J. C. (1965) *Nature* **207**, 28–30
26. Tilton, R. F., jr., Kuntz, I. D., jr., and Petsko, G. A. (1984) *Biochemistry* **23**, 2849–2857
27. Dong, A., Huang, P., Zhao, X. J., Sampath, V., and Coughey, W. S. (1994) *J. Biol. Chem.* **269**, 23912–23917
28. Schoenborn, B. P. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 4195–4199
29. Nakagawa, T., Hama, T., Nishimura, S., Uruga, T., and Kito, Y. (1994) *J. Mol. Biol.* **238**, 297–301
30. Jorgensen, K., Ilsen, J. H., Mortensen, O. G., and Zuckermann, M. J. (1993) *Chem. Phys. Lipids* **65**, 205–216
31. Vero, J. A., and Hunt, G. R. A. (1985) *Chem. Biol. Interact.* **54**, 337–348
32. Akerfeldt, K. S., Lear, J. D., Wasserman, Z. R., Chung, L. A., and DeGrado, W. F. (1993) *Acc. Chem. Res.* **26**, 191–197
33. Fernandez-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J.-L. R., Ferragut, J. A., and Gonzalez-Ros, J. M. (1994) *Biochemistry* **33**, 4065–4071
34. Forman, S. A., Miller, K. W., and Yellen, G. (1995) *Mol. Pharmacol.* **48**, 574–581
