Structure of the Transmembrane Cysteine Residues in Phospholamban

I.T. Arkin[1,*], P.D. Adams[2], A.T. Brünger[3], S. Aimoto[4], D.M. Engelman[2], S.O. Smith[2]

[1]Department of Cell Biology, Yale University, New Haven, CT 06520, USA
[2]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA
[3]Howard Hughes Medical Institute, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520, USA
[4]Institute for Protein Research, Osaka University, Osaka 565, Japan

Abstract. Phospholamban, a 52-residue membrane protein, associates to form a pentameric complex of five long α-helices traversing the sarcoplasmic reticulum membrane of cardiac muscle cells. The transmembrane domain of the protein is largely hydrophobic, with only three cysteine residues having polar side chains, yet it functions as a Ca^{2+}-selective ion channel. In this report, infrared spectroscopy is used to probe the conformation of the three cysteine side chains and to establish whether the free S-H groups form intrahelical hydrogen bonds in the pentameric complex. Vibrational spectra of a transmembrane peptide were obtained which corresponded to the transmembrane domain of wild-type phospholamban and three peptides each containing a cysteine $\Rightarrow$ alanine substitution. The observed S-H frequencies argue that each of the sulfhydryl groups is hydrogen-bonded to an i-4 backbone carbonyl oxygen. Electrostatic calculations on a model of phospholamban based on molecular dynamics and mutagenesis studies, show that the sulfhydryl groups may significantly contribute to the electrostatic potential field of the protein.

Key words: Infrared spectroscopy — Membrane protein — Sulfhydryl — Hydrogen-bonding

Introduction

The mechanisms by which ion channels control ion conductance and specificity are poorly understood. The problem stems in part from the lack of high resolution structures of channel proteins. Two detailed molecular descriptions of ion conductance pathways come from the structures of gramicidin and the acetylcholine receptor. These two proteins have extremely different structures, yet share some common features that provide insights into questions of mechanism. One of the most striking similarities is the role of polar and hydrophobic residues in modulating ion conductance.

In gramicidin, a 15-residue antibiotic peptide secreted by *Bacillus brevis,* the alternating pattern of D and L amino acids yields a unique secondary structure termed the β-helix (Ketchm, Hu & Cross, 1993; Langs et al., 1991). Monomeric peptides form head-to-head dimers in membranes to create a large intra-helix pore which permits ions to transverse the lipid bilayer. The amino acid side chains are all oriented towards the surrounding lipids leaving the pore lined with only the polar backbone carbonyl and amide groups. These groups make the channel cation selective. Roux and coworkers were able to correlate the translocation barrier of a cation through the ion pore to the coordination energy of the ion by the carbonyl oxygens and two water molecules (Roux & Karplus, 1994). Furthermore, there are four tryptophan residues in the sequence that serve to stabilize the channel conformation of the peptide and influence cation conductance (Hu & Cross, 1995; Hu, Lazo & Cross, 1995). Substitution of phenylalanine for tryptophan incrementally decreases conductance (Tredgold et al., 1977; Van Mau et al., 1988; Becker et al., 1991). Based on NMR studies of the structure and dynamics of the tryptophan side chains, Cross and coworkers suggested that the tryptophan dipoles stabilize cations in binding sites near the entrance to the ion channel and reduce the energy associated with crossing the potential barrier at the bilayer center (Hu & Cross, 1995).

---

* *Present address:* Howard Hughes Medical Institute, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Correspondence to: S.O. Smith

Electron cryo-crystallographic studies of 2D crystals of the acetylcholine receptor from *Torpedo marmorata* have led to a molecular structure at 9 Å resolution (Unwin, 1993). The structure is formed from five similar subunits each contributing a transmembrane helix (designated M2) to create a central ion pore. Upon exposing the channel to acetylcholine, the M2 transmembrane helix is postulated to rotate and disrupt a tight central ring of leucine residues (L251) that has blocked the ion pathway (Unwin, 1995). In its place, the polar groups S252, S248 and T244 are thought to line the ion permeation path. In a fashion similar to that of tryptophan in gramicidin, mutation of T244 in the acetylcholine receptor has dramatic effects on ion conductance. Mutation of T244 can either enhance or restrict the flow of ions through the ion channel depending on the volume of the substituted residue (Imoto et al., 1991; Villarroel et al., 1991).

In this report, we describe the structure of the polar groups in the phospholamban pentameric complex. Phospholamban is a 52 residue noncovalent homopentameric membrane protein found in the cardiac sarcoplasmic reticulum (Tada & Kadoma, 1989). Its function is the regulation of the resident Ca^{2+} ATPase. Phospholamban's affinity towards the Ca^{2+} ATPase is reduced upon phosphorylation suggesting that regulation may occur by way of an inhibitory association between the two proteins (James et al., 1989). In addition, phospholamban has been shown to be a Ca^{2+} selective ion channel in in vitro measurements (Kovacs et al., 1988). Ion conductance by phospholamban may contribute to Ca^{2+} ATPase regulation by counteracting the Ca^{2+} current generated by the pump. In this study, we further explore the possibility that channel activity is involved in Ca^{2+} ATPase regulation by investigating the structure and potential role of the polar residues in the phospholamban pentamer.

Structurally, phospholamban has been modeled as a left-handed coiled-coil of five long helies (Adams et al., 1995; Arkin et al., 1994, 1996). The cytosolic region of the helical bundle contains the two residues (S16 and T17) that are phosphorylated in response to β-adrenergic stimulation (Tada & Kadoma, 1989). Specific interactions between transmembrane helices hold the complex together and form a pore through the bilayer (Arkin et al., 1994; Adams et al., 1995). Interspersed between the hydrophobic residues in the transmembrane domain of phospholamban are three cysteine residues (C36, C41 and C46). These are the only polar residues in the transmembrane sequence of the protein and are conserved in all phospholamban sequences. The cysteine residues do not form disulfide bonds (Fujii et al., 1989) and do not appear to be involved in pentamer formation.

Vibrational spectroscopy is a useful tool to study the local environments and structure of sulfhydryl groups. The sulfhydryl group resonates in a relatively transparent region of the vibrational spectrum of proteins (2500–2600 cm^{-1}) (Patai, 1974). Thomas and coworkers have shown in an extensive study of model mercaptan compounds that the S-H frequency is highly sensitive to the nature and strength of hydrogen bonding (Huimin & Thomas, 1992; Li et al., 1992). Sulfhydryl groups dissolved in a solvent incapable of hydrogen-bonding resonate at 2590–2580 cm^{-1}. However, when hydrogen-bonded to water or strongly hydrogen-bonded to carbonyl oxygens, the S-H vibrations shift to 2578–2569 or 2570–2525 cm^{-1}, respectively (Huimin & Thomas, 1991).

To establish whether the free SH groups in phospholamban are hydrogen-bonded, comparisons were made between spectra of the wild type transmembrane peptide (PLBTM) and three peptides each containing a cysteine $\Rightarrow$ alanine substitution (PLB C36A, PLB C41A and PLB C46A). Based on the observed amide I and II frequencies and dichroic ratios, we show that these mutations do not cause structural changes in the protein. These observations have enabled us to selectivity determine the hydrogen-bonding state of each of the sulfhydryl groups and constrain the χ_1 and χ_2 torsion angles which define the orientation of the S-H bond vector.

Finally, electrostatic calculations were undertaken based on a knowledge of the side chain geometry of the three cysteine residues. The purpose behind this effort was an attempt to understand the contribution of the sulfhydryl groups to the electrostatic field that exists within the central pore formed by the pentameric complex.

Materials and Methods

PROTEIN PURIFICATION AND RECONSTITUTION

Peptides were synthesized using standard t-Boc chemistry on a solid phase support, cleaved from the resin with HF and lyophilized. The peptides were 28-residues in length, corresponding to the C-terminal transmembrane domain of the wild-type protein, residues 25–52. The lyophilized peptides were dissolved in 1 ml of trifluoroacetic acid (final concentration *ca.* 4 mg/ml) and immediately injected onto a 5 ml POROS-R1 reverse phase high performance liquid chromatography column (Perceptive Biosystems, Cambridge, MA) equilibrated with 95% H$_2$O, 2% acetonitrile and 3% 2-propanol. Peptide elution was achieved with a linear gradient to a final solvent composition of 5% H$_2$O, 38% acetonitrile and 57% 2-propanol. All solvents contained 0.1% trifluoroacetic acid. Fractions containing peptides were then lyophilized and assessed for purity by amino acid analysis and mass spectrometry. Minor contaminants, peptides that were truncated by 1–2 amino acids, were largely removed by HPLC. The lyophilized peptides were dissolved with a solution of 20% octyl β-glucoside (Sigma, St. Louis, MO) to a final concentration of 4 mg/ml. A 0.5 ml solution containing 20 mg/ml dimyristoylphosphocholine (DMPC, Avanti Polar Lipids, Alabaster, AL) and 10% octyl β-glucoside was added to the protein and reconstitution of the peptides into the phospholipids was achieved by exhaustive dialysis into a buffer containing 1 mM Tris-HCl pH 7.4 (Sigma, St. Louis, MO). The resulting lipid to protein ratios ranged between 1:100 and 1:20.

FTIR SPECTROSCOPY

After dialysis, solutions of the peptides (400 µl containing roughly 0.3 mg/ml of peptide and 10 mg/ml of lipid) were spun down (178,000 × g for 2 hr, Beckman Airfuge, Palo Alto, CA) and resuspended in either 75 µl of H_2O or D_2O (>99.9%, Cambridge Isotope Laboratories, Andover, MA). Two rounds of freeze-thaw-sonication ensured homogeneity of the samples. Finally, the samples were dried down on a Ge infrared window (13 × 2 mm, Grasbey Specac, Kent, UK). FTIR spectra were recorded on a Nicolet Magna 550 spectrometer (Madison, WI) purged with N_2 and equipped with an MCTA detector; 1,000 interferograms at a spectral resolution of 4 cm^{-1} were averaged for each sample. Four different samples were prepared and studied for each of the peptides, yielding reproducible results. Attenuated total reflection ATR-FTIR spectra were obtained and processed as described previously (Arkin et al., 1995).

FTIR DATA PROCESSING

Interferograms were processed in an identical manner, using single point zero filling and Happ-Genzel apodization, followed by automatic base line correction of the resulting spectra. The S-H frequency is extremely weak in the IR spectrum and consequently slight differences are often observed in the baseline position of the corrected spectrum. For comparative purposes, the spectra in the S-H region (2590–2530 cm^{-1}) are normalized with respect to the peptide concentration by taking the ratios of the amide I band area.

ELECTROSTATIC CALCULATIONS

All calculations were carried out on the model of phospholamban arrived at by molecular dynamics and mutagenesis studies previously described (Arkin et al., 1994; Adams et al., 1995). Hydrogen atoms were introduced using the CHARMM22 parameter set (Brooks et al., 1983) with electrostatic and van der Waals energy terms enabled using the program X-PLOR (Brünger, 1992), in order to insure energetically reasonable initial positions for the hydrogen atoms. The model was subsequently subjected to 1,000 steps of Powell energy minimization (Powell, 1977).

Electrostatic calculations were carried out using the program DELPHI (QDIFF) that solves the nonlinear Poisson-Boltzmann equation (Nicholls & Honig, 1991). The linear part of the Poisson-Boltzmann equation was solved using 2,000 iterations, while 3,000 iterations were used to solve the nonlinear portion of the equation. This number of iterations was found to be sufficient for convergence. The only other parameter that was different from default values was the outer dielectric constant which was set to a value 2.0. Charges were taken from the CHARMM22 all atom parameter set (Brooks et al., 1983).

Results and Discussion

AMIDE I AND II VIBRATIONAL MODES: SECONDARY STRUCTURE AND ORIENTATION

The role of the transmembrane cysteine residues was first investigated through mutagenesis studies by Mac-
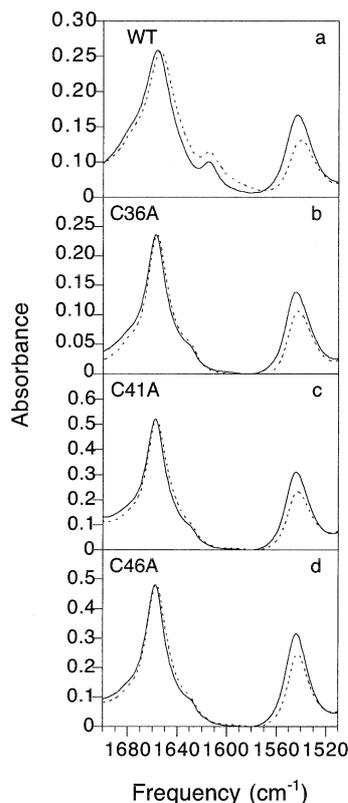

Fig. 1. FTIR spectra in the region of the amide I and amide II vibrational modes of phospholamban. DMPC bilayers containing the transmembrane phospholamban peptides were dried down from H_2O (continuous line) or D_2O (broken line). (*a*) PLBTM, wild-type transmembrane peptide; (*b*) PLB C36A; (*c*) PLB C41A; (*d*) PLB C46A.

Lennan and coworkers (Fujii et al., 1989). They found that replacing each cysteine with an alanine had no effect on oligomerization as assayed by SDS-polyacrylamide gel electrophoresis at 25°C. These studies were the first to suggest that the cysteine residues are not involved in disulfide bond formation. The first FTIR measurements in this study were directed at showing that the secondary structures and membrane incorporation of the peptides containing cysteine $\Rightarrow$ alanine substitutions were the same as wild type.

The amide I and amide II vibrational modes are sensitive to and diagnostic of secondary structure in proteins (Braiman & Rothschild, 1988). Figure 1 presents the amide I-II region of the phospholamban peptides that have been reconstituted into DMPC bilayers. The amide I and amide II bands are centered at 1655 cm^{-1} and 1543 cm^{-1}, respectively, indicative of high helical content (Braiman & Rothschild, 1988). The observation that the amide I and amide II vibrational modes are nearly identical in both frequency and shape in all of the peptides argues that the secondary structures are similar. In the case of the wild-type peptide, two backbone carbonyls were isotopically-enriched with ^{13}C during synthesis

which resulted in an additional amide I mode at 1614 cm^{-1} (Tadesse, Nazarbaghi & Walters, 1991). The position of this isotope-shifted band previously allowed us to establish that the local structure of the phospholamban transmembrane domain is α-helical between residues L39 and L42 (Ludlam et al., 1996).

Figure 1 also presents the results of exchanging the solvent from H$_2$O (continuous line) to D$_2$O (broken line). Comparison of the integrated intensities of the IR bands shows a slight reduction (less than 23%) of the amide II peak upon D$_2$O exchange in each of the four peptides studied. This observation argues that the amide protons are largely protected from exchange, consistent with a transmembrane orientation for the four peptides.

Additional data supporting the conclusion that the peptides have a transmembrane orientation are derived from measurements of the orientation of the peptides in lipid bilayers by ATR-FTIR. In these experiments, the orientation of the backbone C = O groups is determined from the relative absorption of infrared light polarized parallel or perpendicular to the C = O transition dipole moment. The dichroic ratio, R^{ATR}, defined as $A_{II}/A_{\perp}$, can be related to the orientation of the helix axis (Braiman & Rothschild, 1988). As seen in Fig. 2, all of the peptides exhibit significant dichroism of the amide I mode (3.1 < RATR < 3.3). These high dichroic ratios translate into similar transmembrane orientations for the four peptides. Together with the mutagenesis results, the FTIR measurements provide support for the conclusion that the overall structure of the four peptides studied is the same.

SULFHYDRYL VIBRATIONAL MODES: HYDROGEN-BONDING AND CONFORMATION

Figure 3 presents FTIR spectra in the region where the cysteine sulfydryl vibrational mode is observed (2590–2530 cm^{-1} (Patai, 1974)) for the four different peptides (*see* Table). First, it should be pointed out that the extinction coefficient of different sulfhydryl groups in the IR spectrum can vary dramatically depending on the degree of polarization (Alben, Bare & Bromberg, 1974; Bare, Alben & Bomberg, 1975). Sulfhydryls located in a nonhydrogen bonding solvent have an extinction coefficient much smaller than those that are hydrogen-bonded. Therefore, direct quantitative assessment and peak identification may be difficult. For this reason we have examined spectra of the three different mutant peptides each lacking one of the sulfhydryl groups. As all of the spectra appear similar both in absorption magnitude and in frequency, we would expect that the intensities of the different sulfhydryl absorption would be similar, as seen in Fig. 3.

The wild-type PLBTM peptide exhibits a broad unresolved S-H vibration centered at 2563 cm^{-1} that results
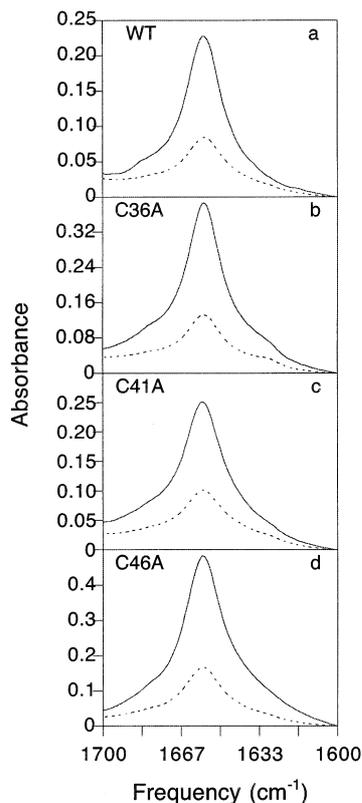

Fig. 2. ATR-FTIR spectra in the region of the amide I vibrational mode of phospholamban in DMPC bilayers. Spectra were obtained using parallel (continuous line) and perpendicular polarized light (broken line). (*a*) PLBTM, wild-type transmembrane peptide; (*b*) PLB C36A; (*c*) PLB C41A; (*d*) PLB C46A.

from the three transmembrane cysteine residues (Fig. 3*a*). Removal of each cysteine individually by substitution to an alanine yields in an S-H band corresponding to the two remaining cysteine residues. The two S-H vibrations are still unresolved, yet the shift of the band relative to wild-type allows us to estimate the S-H frequency of the substituted cysteine. For example, the S-H frequency of the PLB C36A peptide has shifted to 2556 cm^{-1} (Fig. 3*b*) arguing that the frequency of the C36 S-H vibration is higher than the wild-type frequency of 2560 cm^{-1}. A similar conclusion can be drawn for the C46 S-H vibration since the PLB C46A peptide also exhibits a band below the position of wild type. In contrast, the C41 S-H vibrational frequency must be below 2563 cm^{-1} since the S-H band in PLB C41A is above the wild-type frequency. Furthermore, the fact that the sulfhydryl modes are all unresolved indicates that they are all very similar. These results directly show that the cysteine residues are free and not involved in disulfide bond formation.

Sulfhydryl groups that are hydrogen-bonded to carbonyl oxygens are a common occurrence in soluble pro-
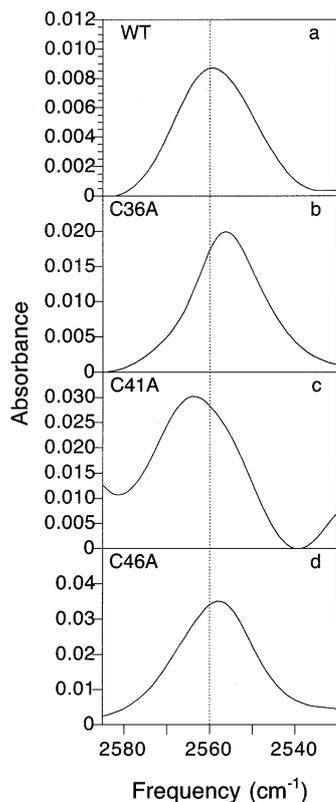
Fig. 3. FTIR spectra in the region of the sulfhydryl S-H stretching mode of phospholamban in DMPC bilayers. (*a*) PLBTM, wild-type transmembrane peptide; (*b*) PLB C36A; (*c*) PLB C41A; (*d*) PLB C46A. The sulfhydryl bands of PLBTM, PLB C36A, PLB C41A and PLB C46A are centered at 2562.7 cm^{-1} (σ = 0.16), 2556.1 cm^{-1} (σ = 0.926), 2564.3 cm^{-1} (σ = 3.511) and 2558.1 cm^{-1} (σ = 0.233), respectively (σ represents standard deviation).

Table. Sulfhydryl vibrational frequencies observed in the wild-type phospholamban transmembrane domain and the three cysteine-to-alanine replacement peptides

Peptide	Sulfhydryl vibrational frequency
Wild type	2560 cm^{-1}
C36A	2556 cm^{-1}
C41A	2557 cm^{-1}
C46A	2566 cm^{-1}

teins. Cohen and coworkers have surveyed the protein data bank and found that 72% of all reduced cysteine residues in proteins are involved in hydrogen bonding (Gregoret et al., 1991). Of these residues, 58% are involved in hydrogen bonding to carbonyl oxygens at position i-4 of an α-helix. In membrane proteins, where the structural data base is far smaller, we identified six instances of sulfhydryl hydrogen bonding in the photosynthetic reaction center structure (Deisenhofer & Michel,

1989). Each of these (C92 and C129 in the L chain, C157, C210, and C296 in the M chain and C201 in the H chain) formed hydrogen bonds to i-4 carbonyl oxygens. Interestingly, the sulfhydryl of C296 in the M chain has an additional bifurcating hydrogen bond to the carbonyl oxygen of Y196 (M chain) that resides on a loop connecting two adjacent helices. A low sulfhydryl vibrational frequency (indicating a strong hydrogen bond to a carbonyl oxygen) in a membrane protein has been seen previously by Thomas and coworkers (Li et al., 1993) in the φ6 bacteriophage. The fact that the sulfhydryl groups tend to hydrogen-bond to the i-4 carbonyl oxygens in the bilayer is especially likely since very few other hydrogen-bond acceptors reside in the low dielectric membrane environment.

The sulfhydryl vibrational frequencies observed for all of the cysteine residues are similar to frequencies obtained by Thomas and coworkers (Huimin & Thomas, 1991) for mercaptans in carbonyl hydrogen bonding solvents (e.g., dimethylacetamide and dimethylformamide). The differences between the individual frequencies can be attributed to either the strength of the hydrogenbond or to differences in the χ_1 and χ_2 torsion angles which can have an effect upon the S-H frequency of up to 10 cm^{-1} (Li et al., 1992). However, variations of χ_1 and χ_2 are limited when one considers the location of possible hydrogen-bond acceptors. If the S-H...O hydrogen bond is constrained to be less than 2.5Å, then the only carbonyl oxygen that would serve as a hydrogen-bond acceptor is at the i-4 position of the same helix (S-H...O = 1.6–1.8 Å). Moreover, the χ_1 and χ_2 angles predicted for intrahelical hydrogen bond are energetically favored over the angles needed for forming interhelical hydrogen bonds (Adams et al., 1995). The closest interhelical carbonyl oxygens to the three cysteine residues are at S-H...O distances of 5.6 Å (C36), 6.6 Å (C41) and 2.83 Å (C46), if one does not constrain the values of χ_1 and χ_2 (Ponder & Richards, 1987; Schrauber, Eisenhaber & Argos, 1993). Therefore, it is expected that variations of the side chain torsion angles are not major contributors to the observed differences in the S-H frequencies. This argues that all of the thiol groups in phospholamban are hydrogen-bonded to backbone carbonyl oxygens at position i-4 of the helix, as shown in Fig. 4.

A model obtained by molecular dynamics simulations reported previously (Adams et al., 1995) predicts hydrogen bonding for all of the 15 cysteine residues in the phospholamban pentamer. Furthermore, the hydrogen acceptor in all cases is the backbone carbonyl at position i-4. This agrees with the pattern of S-H hydrogen bonding observed in protein crystal structures and clearly shows that the energy associated with hydrogen bonding of the free S-H group in a hydrophobic environment is sufficient to stabilize the observed structures.
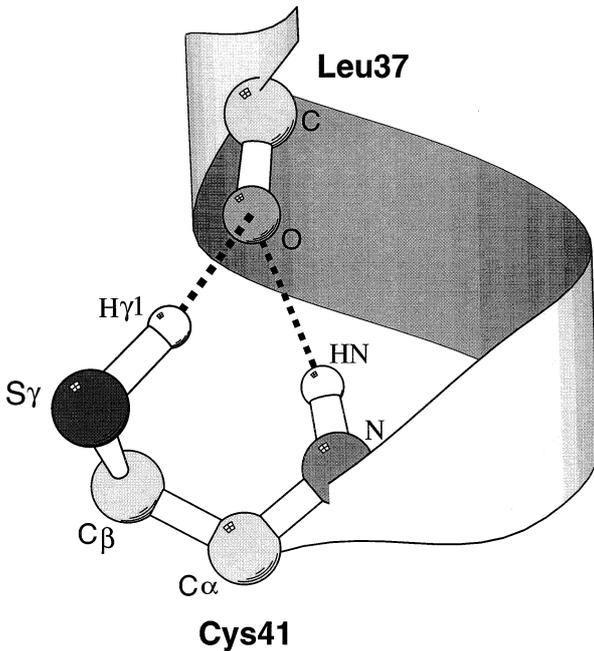
Fig. 4. Molecular model showing the sulfhydryl group of Cys 41 hydrogen bonding back to the carbonyl oxygen of Leu 37. This hydrogen-bonding pattern is characteristic of cysteine residues in α-helices and was previously predicted using computational approaches to model the phospholamban transmembrane domain (Adams et al., 1995). The figure was made using the program Molscript (Per Kraulis).

INFLUENCE OF THE SULFHYDRYL GROUPS ON THE ELECTROSTATIC FIELD

Since the cysteine residues do not appear to have an essential structural role in phospholamban, the question then arises as to the possible function of the large number of sulfhydryl groups in this ion channel. Unlike the acetylcholine receptor, in which the polar residues appear to line the ion channel, the cysteine residues in phospholamban are distributed around the transmembrane helix. Mutagenesis and S-H exchange measurements indicate that C41 is oriented towards the surrounding lipids and C46 is packed in the helix interface (Arkin et al., 1994; Arkin et al., 1996). C36 is the only cysteine that contributes to the accessible surface of the channel, although S-H exchange measurements indicate that the sulfhydryl is protected. One possible hypothesis for the role of the three cysteine residues is that the particular electrostatic properties of the sulfhydryl groups help shape the overall electrostatic field of the ion channel. The electrostatic field is likely to be a contributing element in establishing the specificity and conductivity of the channel. This idea was explored by calculating the electrostatic potentials in the channel using the structural model of phospholamban (Arkin et al., 1994; Arkin et al., 1996).

It is important to state that the electrostatic calcula-

tions were undertaken for a *model* of phospholamban and not a structure determined by crystallographic or NMR methods. Furthermore, electrostatic calculations such as those undertaken in this study are inherently simplistic in that they assume uniform dielectric constants throughout. Nevertheless, the calculations directly address the possibility that the three cysteine residues play a functional role in phospholamban.

Figure 5 presents difference electrostatic maps between the wild-type transmembrane domain and models where the cysteine χ_2 angles were changed by 180°. The contours are at levels of ±10 kT and show that changes can be as large as 80 kT. Such large differences from small changes in the protein are most likely caused by the low dielectric nature of the lipid environment, and in this case, the effects are augmented by the pentameric state of the channel. Moreover, changes in the electric field within a channel are observed even for those cysteines located in the helix (C46) and lipid (C41) interfaces. The idea that minor conformational changes in a protomer may contribute to a major electrostatic change in a complex is probably not unique to phospholamban, as most ion channels are based on repetitive elements.

One of the implications that can be drawn from the electrostatic calculations is that it may be experimentally difficult to detect a subtle structural change involved in channel gating. Small differences in χ_2 torsion angles are nearly impossible to detect by any other method except FTIR and possibly the solid-state NMR approaches developed by Cross and Opella (1994). Protons are not seen in x-ray crystallographic electron density maps at >1 Å resolution. Furthermore, this study clearly indicates that the results of electrostatic calculations may be misleading if the exact positions of all of the atoms, including hydrogens and associated water, are not known.

CONCLUSIONS

Infrared measurements of a transmembrane peptide corresponding to wild-type phospholamban along with three cysteine ⇒ alanine substituted peptides has argued that all of the sulfhydryl groups in phospholamban are hydrogen-bonded to i-4 carbonyl oxygens. Difference electrostatic maps imply that large changes in the electrostatic field within the central phospholamban pore may result from small changes in the cysteine χ_2 rotamer angle. This observation suggests that both the distribution and orientation of polar groups (such as serine, threonine, cysteine or tryptophan) in the transmembrane domain of the channel protein are important in establishing the electrostatic profile in the ion pore and in controlling the energy barriers for ion translocation, even when these groups are not located in the channel itself. This emphasizes the important role of polar residues in
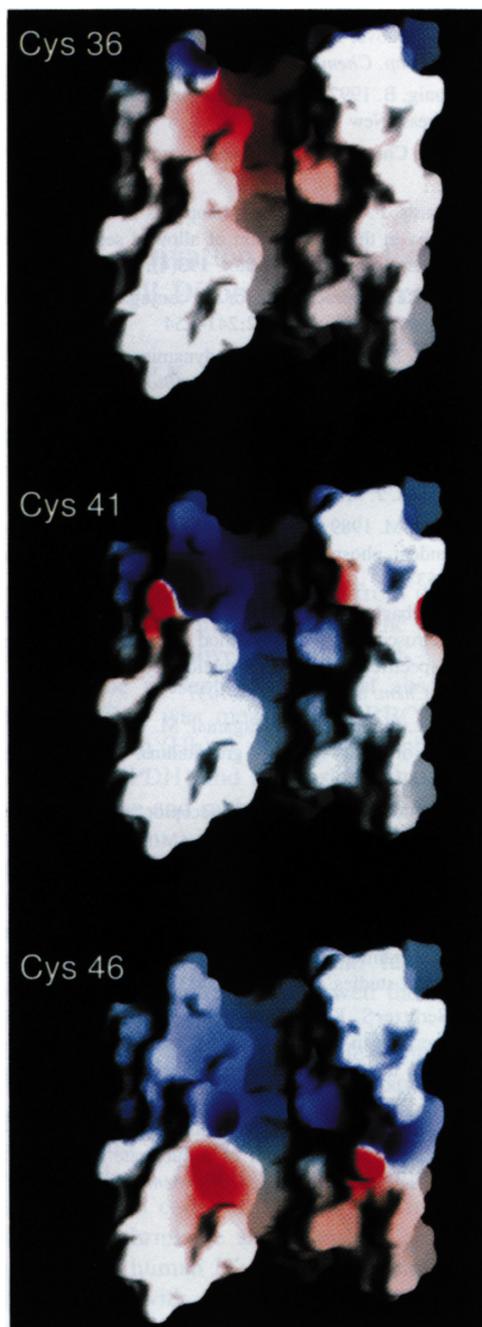
Fig. 5. Difference electrostatic maps of the phospholamban transmembrane domain as a function of the cysteine side chain χ_2 torsion angles. The potential fields were calculated using the program DELPHI23 and displayed using the program GRASP (Nicholls & Honig, 1992). Differences are defined as wild-type channel minus altered state. The three differences displayed correspond to the three different cysteine side chains with their χ_2 angles rotated by 180°. Blue represents a positive change in electrostatic potential, while red represents a negative change. Values range between ±10 kT.

regulating ion conductance and selectivity. These results also suggest that it may be difficult to draw correlations between altered conductivity and the location of polar residues in the ion pore based on site-directed (Akabas et al., 1994) or cysteine-scanning mutagenesis (Wu & Kaback, 1994).

References

Adams, P., Arkin, I., Engelman, D., Brünger, A. 1995. Computational searching and mutagenesis suggest a structure for the pentameric transmembrane domain of phospholamban. *Nature Struct. Biol.* **2(2):**154–162

Akabas, M., Kaufmann, C., Archdeacon, P., Karlin, A. 1994. Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the α subunit. *Neuron* **13(4):**919–927

Alben, J., Bare, G., Bromberg, P. 1974. Sulfhydryl groups as a new molecular probe at the α1 β1 interface in haemoglobin using Fourier transform infrared spectroscopy. *Nature* **252(5485):**736–738

Arkin, I., Adams, P., MacKenzie, K., Lemmon, M., Brünger, A., Engelman, D. 1994. Structural organization of the pentameric transmembrane α-helices of phospholamban, a cardiac ion channel. *EMBO J.* **13(20):**4757–4764

Arkin, I., Fisher, L., MacKenzie, K., Aimoto, S., Engelman, D., Smith, S. 1996. Mapping the lipid exposed surfaces of membrane proteins. *Nature Struct. Biol.* **3:**240–243

Arkin, I., Rothman, M., Ludlam, C., Aimoto, S., Engelman, D., Rothschild, K., Smith S. 1995. Structural model of the phospholamban ion channel complex in phospholipid membranes. *J. Mol. Biol.* **248(4):**824–834

Bare, G., Alben, J., Bromberg, P. 1975. Sulfhydryl groups in hemoglobin. A new molecular probe at the α1 β1 interface studied by Fourier transform infrared spectroscopy. *Biochemistry* **14(8):**1578–1583

Becker, M., Greathouse, D., Koeppe, R.E., Andersen, O. 1991. Amino acid sequence modulation of gramicidin channel function: effects of tryptophan-to-phenylalanine substitutions on the single-channel conductance and duration. *Biochemistry* **30(36):**8830–8839

Braiman, M., Rothschild, K. 1988. Fourier transform infrared techniques for probing membrane protein structure. *Annu. Rev. Biophys. Biophys. Chem.* **17:**541–570

Brooks, B., Bruccoleri, R., Olafson, B., States, D., Swaminathan, S., Karplus, M. 1983. CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. *J. Comp. Chem.* **4:**187–217

Brünger, A. 1992. X-PLOR Version 3.1 first edition. Yale University Press, New Haven, CT

Cross, T., Opella, S. 1994. Solid state NMR structural studies of peptides and proteins in membranes. *Curr. Opin. Struct. Biol.* **4(4):**574–581

Deisenhofer, J., Michel, H. 1989. Nobel lecture. The photosynthetic reaction centre from the purple bacterium Rhodopseudomonas viridis. *EMBO J.* **8(8):**2149–2170

Fujii, J., Maruyama, K., Tada, M., MacLennan, D. 1989. Expression and site-specific mutagenesis of phospholamban. Studies of resi-

dues involved in phosphorylation and pentamer formation. *J. Biol. Chem.* **264(22):**12950–12955

Gregoret, L., Rader, S., Fletterick, R., Cohen, F. 1991. Hydrogen bonds involving sulfur atoms in proteins. *Proteins* **9(2):**99–107

Hu, W., Cross, T. 1995. Tryptophan hydrogen bonding and electric dipole moments: functional roles in the gramicidin channel and implications for membrane proteins. *Biochemistry* **34(43):**14147–14155

Hu, W., Lazo, N., Cross, T. 1995. Tryptophan dynamics and structural refinemen in a lipid bilayer environment: solid state NMR of the gramicidin channel. *Biochemistry* **34(43):**14138–14146

Huimin, L., Thomas, G.J. 1991. Cysteine conformation and sulfhydryl interaction in proteins and viruses. 1. Correlation of the Raman S-H band with hydrogen bonding and intramolecular geometry in model compounds. *J. Am. Chem. Soc.* **113:**456–462

Imoto, K., Konno, T., Nakai, J., Wang, F., Mishina, M., & Numa, S. 1991. A ring of uncharged polar amino acids as a component of channel constriction in the nicotinic acetylcholine receptor. *FEBS Lett.* **289(2):**193–200

James, P., Inui, M., Tada, M., Chiesi, M., Carafoli, E. 1989. Nature and site of phospholamban regulation of the Ca^{2+} pump of sarcoplasmic reticulum. *Nature* **342(6245):**90–92

Ketchem, R., Hu, W., Cross, T. 1993. High-resolution conformation of gramicidin A in a lipid bilayer by solid-state NMR. *Science* **261(5127):**1457–1460

Kovacs, R., Nelson, M., Simmerman, H., Jones, L. 1988. Phospholamban forms Ca^{2+}-selective channels in lipid bilayers. *J. Biol. Chem.* **263(34):**18364–18368

Langs, D., Smith, G., Courseille, C., Precigoux, G., Hospital, M. 1991. Monoclinic uncomplexed double-stranded, antiparallel, left-handed β 5.6-helix (increases decreases β 5.6) structure of gramicidin A: alternate patterns of helical association and deformation. *Proc. Natl. Acad. Sci. USA* **88(12):**5345–5349

Li, T., Bamford, D., Bamford, J., Thomas, G.J. 1992. Cysteine conformation and sulfhydryl interaction in proteins and viruses. 2. Normal coordinate analysis of the cysteine side chain in model compounds. *J. Am. Chem. Soc.* **114:**7463–7469

Li, T., Bamford, D., Bamford, J., Thomas, Jr, G. 1993. Structural studies of the enveloped dsRNA bacteriophage phi 6 of Pseudomonas syringae by Raman spectroscopy. I. The virion and its membrane envelope. *J. Mol. Biol.* **230(2):**461–472

Ludlam, C., Arkin, I., Liu, X., Rothman, M., Rath, P., Aimoto, S., Smith, S., Engelman, D., Rothschild, K. 1996. FTIR spectroscopy and site-directed isotope labelling as a probe of the local secondary structure in the transmembrane domain of phospholamban. *Biophys. J.* **70:**1728–1736

Nicholls, A., Honig, B. 1991. A rapid finite difference algorithm utilizing successive over-relaxation to solve the Poisson-Boltzmann equation. *J. Comp. Chem.* **12:**435–445

Nicholls, A., Honig, B. 1992. GRASP a manual first edition. Columbia University Press, New York, NY

Patai, S. 1974. The Chemistry of the Thiol Group. London: John Wiley & Sons

Ponder, J., Richards, F. 1987. Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. *J. Mol. Biol.* **193(4):**775–791

Powell, M. 1977. Restart procedures for the conjugate gradient. *Methods in Mathematical Progress.* **12:**241–254

Roux, B., Karplus, M. 1994. Molecular dynamics simulations of the gramicidin channel. *Annu. Rev. Biophys. Biomol. Struct.* **23:**731–761

Schrauber, H., Eisenhaber, F., Argos, P. 1993. Rotamers: to be or not to be? An analysis of amino acid side-chain conformations in globular proteins. *J. Mol. Biol.* **230(2):**592–612

Tada, M., Kadoma, M. 1989. Regulation of the Ca^{2+} pump ATPase by cAMP-dependent phosphorylation of phospholamban. *Bioessays* **10(5):**157–163

Tadesse, L., Nazarbaghi, R., Walters, L. 1991. Isotopically enhanced infrared spectroscopy: a novel method for examining secondary structure at specific sites in conformationally heterogeneous peptides. *J. Am. Chem. Soc.* **113:**7036–7037

Tredgold, R., Hole, P., Sproule, R., Elgamal, M. 1977. Single channel characteristics of some synthetic gramicidins. *Biochim. Biophys. Acta.* **471(2):**189–194

Unwin, N. 1993. Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* **229(4):**1101–11024

Unwin, N. 1995. Acetylcholine receptor channel imaged in the open state. *Nature* **373:**37–43

Van Mau, N., Trudelle, Y., Daumas, P., Heitz, F. 1988. Mixed monolayers of linear gramicidins and phospholipid. Surface pressure and surface potential studies. *Biophys. J.* **54(3):**563–567

Villarroel, A., Herlitze, S., Koenen, M., Sakmann, B. 1991. Location of a threonine residue in the α-subunit M2 transmembrane segment that determines the ion flow through the acetylcholine receptor channel. *Proc. R. Soc. Lond.* **243(1306):**69–74

Wu, J., Kaback, H. 1994. Cysteine 148 in the lactose permease of *Escherichia coli* is a component of a substrate binding site. 2. Site-directed fluorescence studies. *Biochemistry* **33(40):**12166–12171