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^{23}Na-NMR study of ion transport across vesicle membranes facilitated by phenylalanine analogs of gramicidin

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The transport of Na^+ ions across phosphatidylcholine/phosphatidylglycerol large unilamellar vesicle membranes facilitated by phenylalanine analogs of gramicidin A has been studied using ^{23}Na-NMR spectroscopy. The four analogs studied were Phe^9-, Phe^{11}-, Phe^{13}-and Phe^{15}-gramicidin A. These analogs were found to transport Na^+ ions in the following order Phe^{15} > Phe^{13} > Phe^{11} > Phe^9. The entropy and enthalpy of activation for the transport of Na^+ ions were determined for each analog. A correlation is made between the activation enthalpies and the single channel conductance values of the analogs.

Introduction

Gramicidin is a 15 amino acid linear polypeptide which forms monovalent cation conducting channels in lipid membranes [1,2]. In membranes the gramicidin channel consists of two monomeric β-helices joined by six hydrogen bonds at their NH_2-terminal ends [3–8]. Each monomer exists as a right-handed [9–11], single-stranded β-helix with approx. 6.3 residues per turn and a pitch of about 4.8 Å. The dominant, naturally-occurring analog is gramicidin A. The amino acid sequence of gramicidin A is: formyl-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu ^{10}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-ethanolamine. Gramicidin B, another of the naturally occurring analogs, differs from gramicidin A by having phenylalanine at position 11 instead of tryptophan [12]. The replacement of the tryptophan residues in gramicidin A with phenylalanine results in a reduction of the channel conductance [13–16]. This reduction in single channel conductance, which reflects a modulation of the energy barrier to movement through the channel, is the result of changes in the interaction between the Na^+ ion and the dipole moment along the channel. The dipole moment encountered by the ion, as it enters the gramicidin channel and undergoes translocation through it, is a complex combination of side chain polarity, position of substitution and orientation of the side chains. A precise analysis of the single channel conductance dependence upon the ion-dipole moment interaction would require a three-dimensional structure of each analog incorporated into a membrane environment. Although such a complete description is not available, the effect of position and polarity of single amino acid substitution on the energy barrier to ion transport can be determined with the application metal ion NMR techniques.

The effect of the position of substitution of phenylalanine in the gramicidin A sequence upon ion transport has been obtained by the determination of the activation enthalpy and entropy for the Na^+ ion transport by four phenylalanine analogs using ^{23}NMR spectroscopy. To study the gramicidin facilitated transport of Na^+ ions across vesicle membranes, an aqueous NaCl solution of large unilamelar vesicles, with gramicidin incorporated into them, was used. When a membrane impermeable chemical shift reagent is added to the vesicle solution the internal and external pools of Na^+ ions can be observed by their separate ^{23}Na-NMR signals (Fig. 1). The transport rate through the gramicidin channel can be determined from the ^{23}Na-NMR linewidth (Fig. 2) or with the application of the magnetization inversion transfer technique as a function of gramicidin concentration or temperature [17].

Materials and Methods

Gramicidin B was obtained by the purification of commercial gramicidin D, a mixture of gramicidins A, B and C, obtained from Sigma using previously described methods [18]. The other gramicidin analogs

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were obtained by total peptide synthesis. For the synthesis, Boc-Amino Acid Resins and Boc-Amino Acids were used that were purchased from Peninsula. *N,N*-Dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIPEA), 1,3-dicyclohexylcarbodiimide (DCC), indole and trifluoroacetic acid were 99% pure or better and were purchased from Aldrich. Dichloromethane (DCM) was HPLC grade and was purchased from Fisher. Ethanolamine, *p*-nitrophenolformate and *N*-methylmorpholine were provided by Dr. Roger E. Koeppe, II, Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR. The synthesis of the gramicidin analogs was performed on a Vega 1000 semi-automatic peptide synthesizer. The synthesis was performed for 1 mmol (1–2 g) of Boc-Amino Acid Resin as follows: (1) the resin was washed six times with DCM; (2) deprotection (Boc removal) was achieved by a 10 min treatment with 65% TFA/DCM solution, containing indole at a concentration of 1 mg/ml, to protect the tryptophan side chains; (3) the resin was washed again six times with DCM; (4) a neutralizing wash with 10% DIPEA/DCM was performed twice for 2 min each followed by two washes with DCM, two washes with DMF and finally six washes with DCM; (5) the coupling for all amino acids, except leucine, was achieved by a 10 min reaction with 4 mmol of Boc-Amino Acid and 2 mmol of DC in 5 ml of DCM. For leucine, 2.4 mmol of BOC-Amino Acid and 2.5 mmol of DCC in 10 ml was reacted for 30 min to prevent the possibility of double insertion; (6) this was followed by the addition of 5–7 ml of DMF after which the coupling process was allowed to proceed for another 1–2 h; (7) finally the ninhydrin test [19] was performed to check for the completeness of the coupling reaction. These steps were repeated for each amino acid until the sequence was completed. The polypeptide resin was then dried under vacuum for 24 h.

To reclaim the synthesized gramicidin analog from the resin, the peptide was removed from the resin by adding a 50% solution of methanol and redistilled ethanolamine and heating at 60°C for 48 h. The resin was then filtered and washed with methanol. The filtrate was concentrated and the peptide precipitated by

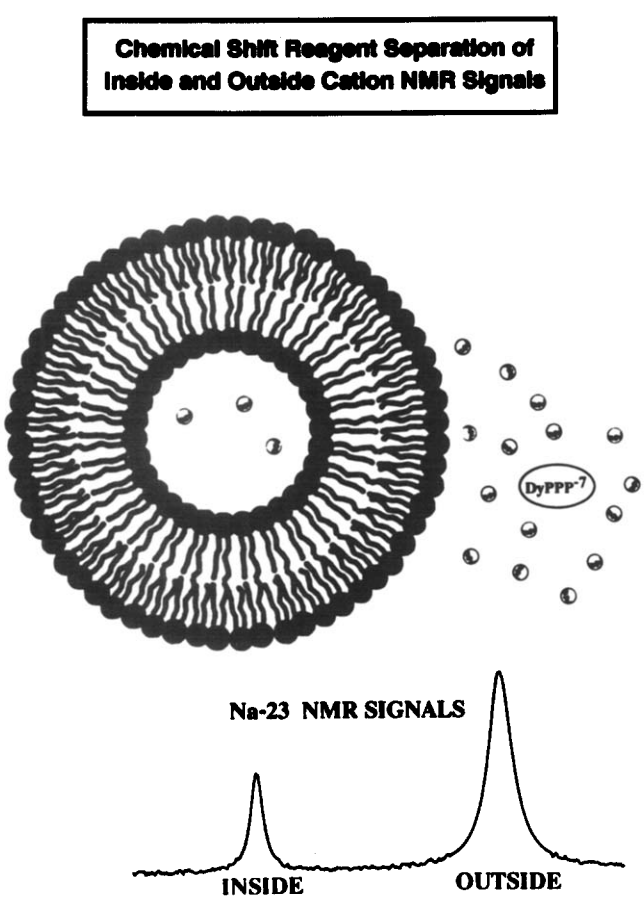

Fig. 1. ^{23}Na-NMR spectrum of vesicle solution showing the inside and outside aqueous Na^+ ion pools.

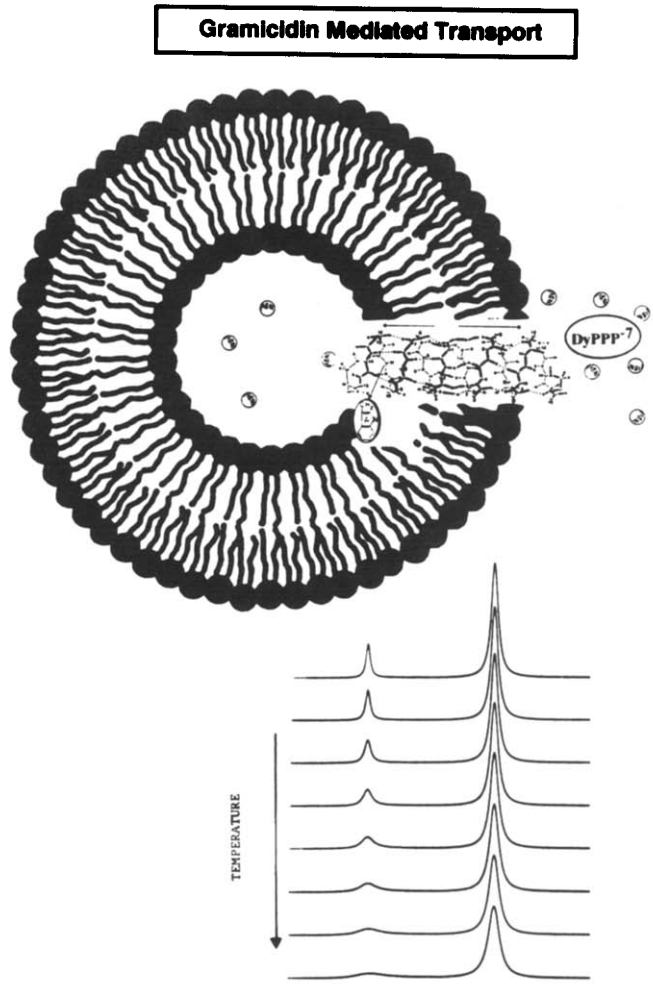

Fig. 2. ^{23}Na-NMR spectrum showing the effect of temperature on linewidth for the vesicles containing gramicidin. As the temperature increases, the transport rate through the channel increases and the linewidth of each ^{23}Na-NMR signal gets broader.

the addition of water. After drying the precipitate, the crude product was purified by HPLC [18]. The Boc group was removed by reacting the Boc-desformyl-gramicidin with 4 M HCl in dioxane at 40°C under nitrogen for 3 h. This mixture was then placed on a AGMP 50 ion exchange column and washed with 500 ml of methanol. The gramicidin was eluted from the column with 200 ml of 2 M NH_4OH in methanol while cooling the column at 4°C. Fractions were collected and the solvent removed under reduced pressure. The desformyl gramicidins were then formulated by reaction with *p*-nitrophenolformate and *N*-methylmorpholine. Each analog was purified on a LH-20 column using methanol as the solvent.

Phosphatidylcholine (PC) (Sigma) and phosphatidylglycerol (PG) (Avanti Polar Lipids) were used to produce large unilamelar vesicles by reverse phase evaporation [20,21]. Each milliter of vesicle solution was produced from a total of 66 μM of lipids in a 4:1 PC/PG molar ratio. The solutions inside and outside of the vesicles contained 100 mM NaCl and were buffered with 10 mM K_2HPO_4/H_3PO_4 to a pH of 8.2. Filtration of the vesicles through 0.4 μm polycarbonate membranes produced a reasonably homogeneous solution of vesicles with an average diameter of 400–500 nm [20]. To incorporate gramicidin into the vesicle membranes, a known concentration (0.5–1 mM) of gramicidin dissolved in TFE was added to the vesicle solution. After agitating the mixture with a vortex mixer, the sample was placed in a constant temperature bath at 65°C for 3 h. Following thermal incubation, the sample was removed from the bath and allowed to attain room temperature. The samples were then diluted 1:1 with an aqueous shift reagent $[Dy(P_3O_{10})_2^{7-}]$ [22] such that the final concentration of the shift reagent on the outside of the vesicle was 5 mM and there was an ionic balance between the inside and outside aqueous vesicle pools. The solutions were stored overnight at 4°C and then allowed to equilibrate to the NMR probe temperature before data acquisition. A vesicle sample without gramicidin was treated in a similar manner and used to correct the gramicidin containing sample for temperature effects other than Na^+ ion exchange between inside and outside aqueous environments.

The ^{23}Na spectra were recorded at 23.65 MHz on a JEOL FX90Q spectrometer using a 10-mm probe with temperature control of ± 0.1°C. Typically, 1000 FIDs were accumulated for each spectrum of each sample. The rate constant for the transport of Na^+ ions through the gramicidin channel was determined as a function of temperature from a measurement of the linewidth at half-height of the ^{23}Na-NMR peak of the Na^+ ions on the inside of the vesicles. The change in linewidth for each sample was corrected by subtracting the linewidth at half-height of the ^{23}Na-NMR peak of the inside Na^+ ion pool of the blank from that of the sample. The linewidth at half-height of the NMR signal is proportional to the square of the total gramicidin concentration [Gr] [17]:

$$1/\tau = \pi \Delta \nu_{1/2} = k[\mathrm{Gr}]^2 \tag{1}$$

where τ is the mean life time of the Na^+ ions on the inside of the vesicles, $\Delta\nu_{1/2}$ is the linewidth at half-height of the inside ^{23}Na-NMR signal and k is the apparent rate constant for the transport process. The apparent rate constant does not represent the true rate constant for transport through the channel. To obtain the true rate constant for the transport process, one must examine the status of gramicidin in the lipid membrane. The ion transporting channel of gramicidin is a dimer that is formed through an equilibrium process represented by:

$$\mathrm{Gr} + \mathrm{Gr} \underset{K_d}{\overset{K_a}{\rightleftharpoons}} \mathrm{Gr}_2 \tag{2}$$

where K_a and K_d are the association and dissociation constants, respectively. At equilibrium, the dimer concentration $[Gr_2]$ is represented by:

$$[\mathrm{Gr}_2] = (K_a/K_d)[\mathrm{Gr}]^2 \tag{3}$$

Assuming that the gramicidin facilitated transport of Na^+ ions across the membrane is directly proportional to the dimer concentration and the Na^+ ion concentration, the mean lifetime of the Na^+ ions on the inside of the vesicles is:

$$1/\tau = \pi \Delta \nu_{\frac{1}{2}} = k_t[\mathrm{Na}^+][\mathrm{Gr}_2] = k_t(K_a/K_d)[\mathrm{Na}^+][\mathrm{Gr}]^2 \tag{4}$$

where k_t represents the rate constant for ion transport. Since the equilibrium constant for the gramicidin dimerization process is extremely large ($K = 1.5 \cdot 10^{14}$ cm^2/mol at 25°C, at a zero limiting voltage on a two-dimensional surface as determined by single channel conductance measurements for gramicidin A in dioleoyllecithin/n-decane membranes [23]) and, therefore, $[Gr_2] \cong [Gr]/2$, the rate constant for the transport process, k_t can be calculated using:

$$k_t = \pi \Delta \nu_t / [\mathrm{Na}^+]([\mathrm{Gr}]/2) \tag{5}$$

Experiments were performed with varying concentrations of gramicidin (the range of lipid/gramicidin ratios covered was 6000:1 to 500:1), different average vesicle size and as a function of shift reagent concentration. Within the limit of experimental error, no effect upon the activation parameters was observed.

The magnetization inversion transfer technique [24–26] was also used to obtain rate constants as a function of temperature to corroborate the activation enthalpy

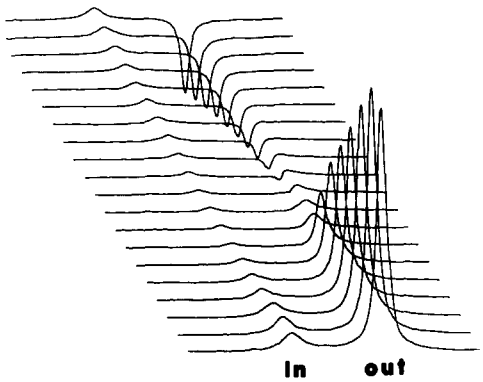

Fig. 3. ^{23}Na magnetization inversion transfer experiment. The outside Na^+ ion signal is inverted and the change in the inside signal intensity is monitored.

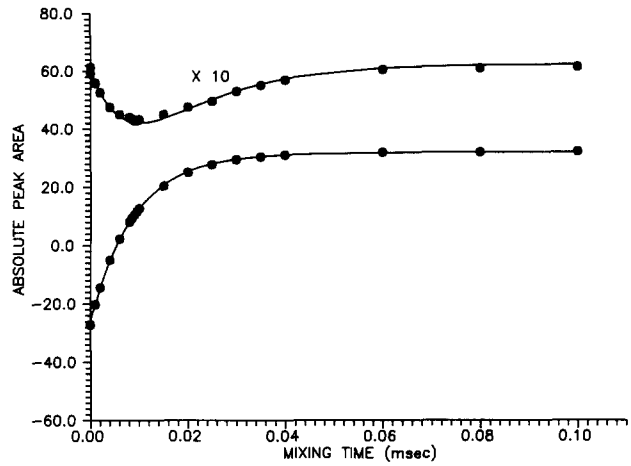

Fig. 4. A magnetization inversion transfer experiment showing the theoretical fit (solid line) to the experimentally determined signal intensity data of the inside ^{23}Na-NMR signal.

and activation entropy values obtained from the linewidth at half-height measurements as a function of temperature. The magnetization inversion transfer technique for a two-site exchange process (e.g., transport of ions from outside of the membrane to the inside) involves magnetically labelling one site by spin population inversion, creating an inverted signal, and then following the effect on the signal intensity of the other site as the inverted signal returns to the initial state by relaxation. If the relaxation and the transport process are of the same time scale, the signal intensity of the non-inverted signal will diminish. The rate constant for the transport can be obtained from the change in the signal intensity with time. This technique has been used in a number of studies of the transport of alkali-metal ions across lipid bilayers [27–32]. The magnetization inversion transfer technique has an advantage over the linewidth technique in that potential difficulties due to effects such as reproducible percentage encapsulation of ions, vesicle size monodispersity, shift reagent variability and relaxation within the channel (the last point has been found not to be a problem even with the linewidth method [33]) are taken into account by the manner in which the complete experiment is performed [32]. Fig. 3 shows a typical magnetization transfer experiment for the $^{23}Na^+$ ions transported through a lipid bilayer by gramicidin A. The method of Muhandiram and McClung [34] was used to extract the rate constant from the magnetization inversion transfer data. An example of the data analysis, where the calculated curve, which depends on the rate constant, is fit to the experimental data is shown in Fig. 4. Gramicidin A was used as a reference standard to monitor spectrometer performance. Good agreement was found between the activation enthalpy for transport determined by the linewidth and the magnetization transfer techniques. With a typical gramicidin A reference sample, for example, the activation enthalpy for transport of Na^+ ions was found to be 8.4 ± 0.4 and 8.5 ± 0.4 kcal/mol determined by magnetization transfer and linewidth techniques, respectively. An activation energy of 7.3 kcal/mol has been obtained for sodium conductance by gramicidin A incorporated into dioleoyllecithin vesicles [35]. Since the linewidth technique requires much less spectrometer time, but provides results comparable to those obtained by the magnetization transfer technique, it was the technique most frequently used. A minimum of two experiments were performed with each analog and in some cases three, when the magnetization transfer and linewidth techniques were used for an analog.

The rate constant (k_t) was determined as a function of temperature for each gramicidin analog to obtain the data necessary for determining the activation enthalpy. Fig. 5 shows a plot of $\ln(k_t/T)$ as a function of $1/T$ for Phe^{11}-Gr A from which the activation enthalpy (ΔH) was obtained. The activation entropy (ΔS) was calculated at 25°C using the absolute rate theory expression for the rate constant and the value of (ΔH)

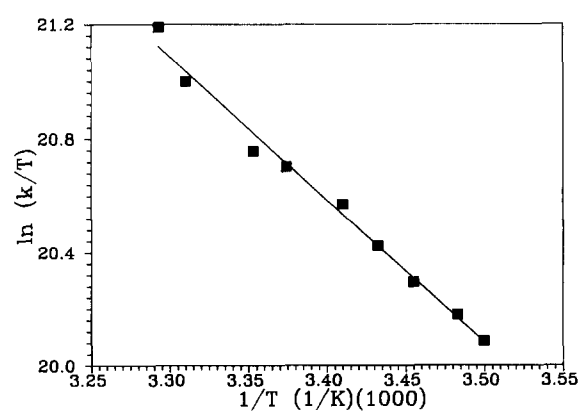

Fig. 5. Plot of $\ln(k_t/T)$ vs. $1/T$ for Phe^{11}-Gr A. The values of k_t were determined using the linewidth method. The activation enthalpy was obtained from the slope of the line.

TABLE I

Activation enthalphy (ΔH), entropy (ΔS), conductance (pS) and channel duration (ms) for Na^+ ion transport by phenylalanine analogs of gramicidin A

Analog	ΔH (kcal/mol)	ΔS (e.u.)	Conductance [a] (pS)	Duration [a] (ms)
Phe^9	10.6±0.3	7.5±2.1	6.0±0.1	1000
				750
Phe^{11}	10.1±0.4	6.5±2.2	8.7±0.3	2300
				2400
Phe^{13}	9.6±0.3	5.5±1.3	11.2±0.2	800
				490
Phe^{15}	9.2±0.2	5.0±1.5	10.9±0.2	790
				790
Gr A	8.5±0.2	4.2±1.2	15.0±0.2	840 [b]

[a] Data taken from Ref. 36.

[b] The variation for Gr A in nine different experiments ranged from 660 to 960 ms.

obtained from the slope of the plot of $\ln(k_t/T)$ vs. $1/T$.

Results and Discussion

The values for the activation enthalpy (ΔH) and activation entropy (ΔS) obtained for each Phe-analog of gramicidin A are contained in Table I. The enthalpies of activation of the analogs, including gramicidin A, decrease in the following order: $Phe^9 > Phe^{11} > Phe^{13} > Phe^{15} >$ Gr A. The maximum single channel conductance of Na^+ ions (1.0 M NaCl) has been determined for the Phe-analogs, Phe^9, Phe^{11}, Phe^{13}, Phe^{15} and gramicidin A [36]. These conductance values are contained in Table I. The analogs can be arranged in the following order of increasing conductance: $Phe^9 < Phe^{11} < Phe^{13} \cong Phe^{15} <$ Gr A. The activation enthalpy, as determined, is a measure of the overall energy barrier for the transport of Na^+ ions through the gramicidin channel from the binding at the channel entrance, translocation through the channel to exiting from the channel. An increase in the activation enthalpy for the ion transport process would be expected to cause a concomitant decrease in the conductance. For the analogs studied, there appears to be a good correlation between the activation enthalpy and single channel conductance for the transport of Na^+ ions through the gramicidin channel. From the determination of the channel-forming potency of these analogs and for Gr A, it has been shown that the overall energy barrier for dimer formation is higher for the Phe analogs than for Gr A [36]. This barrier to channel formation would contribute to the overall barrier to transport. The activation enthalpies for transport obtained for the Phe analogs and Gr A are consistent with this feature of the overall transport process. However, no apparent relation exists between the activation enthalpy or the conductance and the average channel duration, as shown in Table I. Obviously, residue side chains, type and position in the sequence, modify the barrier to ion transport through changes in the ion–dipole moment interaction. The data contained in Table I also reveal that a decrease in the enthalpy barrier to ion transport is accompanied by a decrease in the activation entropy. Further discussion of the significance of the change in activation entropy in terms of channel structure is not possible at this time.

The importance of sequence position and orientation of side chains on conductance has been previously discussed [37–42]. It has been suggested that ion–dipole electrostatic interaction is the dominant type of interaction that determines the channel conductance [37]. The analysis of the single channel conductance of the gramicidin analog containing four phenylalanine residues at positions 9, 11, 13 and 15 instead of tryptophans indicated that the main difference between gramicidin A and the Phe-analog is the consequence of a difference in the height of the energy barriers along the channel [38]. Since no major conformational difference was observed between the Phe-analog and gramicidin A in solution or in lipid medium, it was suggested that the change in conductance was the result of a difference in dipole moment and, hence, a modulation of the transport energy barrier. Theoretical calculations of the energy profile of an ion in the gramicidin channel indicate that the different side chains contribute to the energy in a manner based upon the polar nature of the side chain and side chain position [39]. A precise description of the relationship between the ion–dipole moment interaction and conductance requires a knowledge of the orientation of the side chains with respect to one another in all of the gramicidin analogs incorporated into a lipid environment. This information is not currently available. However, one would expect that replacing tryptophan with the less polar phenylalanine would result in a decrease in the overall ion–dipole moment interaction and, consequently, an increase in the activation enthalpy barrier. This is clearly observed in the comparison of the activation enthalpy and single channel conductance of the Phe-analogs with that of gramicidin A.

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References

1 Meyers, V.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 313–322.
2 Andersen, O. (1983) Biophys. J. 41, 119–133.
3 Urry, D.W. (1971) Proc. Natl. Acad. Sci. USA 68, 672–676.

4 Szabo, G. and Urry, D.W. (1979) Science (Washington, DC) 203, 55–57.
5 Bamberg, E., Apell, H.J. and Alpes, H. (1977) Proc. Natl. Acad. Sci. USA 74, 2402–2406.
6 Weinstein, S., Wallace, B.A., Blout, E.R., Morrow, J.S. and Veatch, W. (1979) Proc. Natl. Acad. Sci. USA 76, 4230–4234.
7 Weinstein, S., Wallace, B.A., Morrow, J.S. and Veatch, W. (1980) J. Mol. Biol 143, 1–19.
8 Urry, D.W., Walker, J.T. and Trapane, T.L. (1982) J. Membr. Biol. 69, 225–231.
9 Arseniev, A.S., Barsukov, I.L., Bystrov, V.F., Lomize, A.L. and Ovchinnikov, Yu.A. (1979) FEBS Lett. 186, 168.
10 Nicholson, L.K. and Cross, T.A. (1989) Biochemistry 28, 9379–9384.
11 Koeppe, R.E.,III, Providence, L.L., Greathouse, D.V., Heitz, F., Trudelle, Y., Purdie, N. and Andersen, O. (1992) Proteins 12, 49–61.
12 Sarges, R. and Witkop, B. (1965) J. Am. Chem. Soc. 87, 2027–2030.
13 Bamberg, E., Noda, K., Gross, E. and Läuger, P. (1976) Biochim. Biophys. Acta 419, 223–228.
14 Prasad, K.U., Trapane, T.L., Busath, D., Szabo, G. and Urry, D.W. (1983) Int. J. Pept. Prot. Res. 22, 341–347.
15 Heitz, F., Spach, G. and Trudelle, Y. (1982) Biophys. J. 39, 87–89.
16 Mazet, J.L. and Andersen, O. (1984) Biophys. J. 45, 263–276.
17 Buster, D.C., Hinton, J.F., Millett, F.S. and Shungu, D.C. (1988) Biophys. J. 53, 145–152.
18 Koeppe, R.E. II, Packowski, J.A. and Whalley, W.L. (1985) Biochemistry 24, 2822–2826.
19 Sarin, R., Kent, S.B.H., Tam, J.P. and Merrifield, R.B. (1981) Anal. Biochem. 117, 147–157.
20 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
21 Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 601, 559–571.
22 Gupta, R.K. and Gupta, P. (1982) J. Magn. Res. 47, 344–350.
23 Bamberg, E. and Läuger, P. (1973) J. Membr. Biol. 11, 177–182.
24 Forsén, S. and Hoffman, R.A. (1963) J. Chem. Phys. 39, 2892–2901.
25 Forsén, S. and Hoffman, R.A. (1964) J. Chem. Phys. 40, 1189–1196.
26 Robinson, G., Kuchel, P.W., Chapman, B.E., Doddrell, D.M. and Irving, M.G. (1985) J. Magn. Reson. 63, 314–319.
27 Riddell, F.G., Arumugam, S. and Cox, B.G. (1987) J. Chem. Soc. Chem. Commun. 1890–1891.
28 Riddell, F.G. and Arumugam, S. (1988) Biochim. Biophys. Acta 945, 65–72.
29 Riddell, F.G. and Arumugam, S. (1989) Biochim. Biophys. Acta 984, 6–10.
30 Riddell, F.G., Arumugam, S. and Patel, A. (1990) Inorg. Chem. 29, 2398–2403.
31 Shungu, D.C. and Briggs, R.W. (1988) J. Magn. Reson. 77, 491–503.
32 Shungu, D.C., Buster, D.C. and Briggs, R.W. (1990) J. Magn. Reson. 89, 102–122.
33 Riddell, R.G and Hayer, M.K. (1985) Biochim. Biophys. Acta 817, 313–317.
34 Muhandrian, D.R. and McClung, R.E.D. (1987) J. Magn. Reson. 71, 187–192.
35 Bamberg, E. and Läuger, P. (1974) Biochim. Biophys. Acta 367, 127–133.
36 Becker, M.D., Greathouse, D.V., Koeppe, R.E. II and Andersen, O. (1991) Biochemistry 30, 8830–8839.
37 Becker, M.D., Sawyer, D.B., Maddock, A.K., Koeppe, R.E. II and Andersen, O. (1989) Biophys. J. 55, 503.
38 Koeppe, R.E. II, Mazet, J.L. and Andersen, O. (1990) Biochemistry 29, 512–520.
39 Heitz, F. Cavach, C., Spach, G. and Trudelle, Y. (1986) Biophys. Chem. 24, 139–141.
40 Etchebest, C. and Pullman, A. (1985) J. Biomol. Struct. Dyn. 2, 859–870.
41 Heitz, F., Daumas, P., Van Mau, N., Lazaro, R., Trudelle, Y., Etchebest, C. and Pullman, A. (1988) in Transport Through Membranes: Carriers, Channels and Pumps (Pullman, A., Jortner, J. and Pullman, B., eds.), Vol. 21, pp. 147–165, Kluwer Academic Publisher, The Netherlands.
42 Koeppe, R.E. II, Andersen, O. and Maddock, A.K. (1988) in Transport Through Membranes: Carriers, Channels and Pumps (Pullman, A., Jortner, J. and Pullman, B., eds.), Vol. 21, pp. 133–145, Kluwer Academic Publisher, The Netherlands.