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Dependence of the Bilayer to Hexagonal Phase Transition on Amphiphile Chain Length[†]

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ABSTRACT: Several series of amphiphiles of increasing chain length were tested for their abilities to modify the L_α–H_{II} transition of dielaidoylphosphatidylethanolamine using differential scanning calorimetry. Acylcarnitines, alkyl sulfates, alkylsulfobetaines, and phosphatidylcholines, with chain lengths between about 6 and 12 carbon atoms, show an increasing capacity to raise the L_α–H_{II} phase transition temperature of phosphatidylethanolamine. This is ascribed to increased partitioning of the added amphiphile from water into the membrane as the chain length increases. Alkyl sulfates and alkyltrimethylammonium bromides have diminished capacities to raise the L_α–H_{II} transition temperature as the chain length is increased from 12 to 16. This is caused by an increase in the hydrophobic portion of the amphiphile leading to a change in the intrinsic radius of curvature and a decrease in the hydrocarbon packing constraints in the H_{II} phase relative to the shorter chain amphiphiles. The L_α–H_{II} transition temperature of phosphatidylethanolamine with acylcarnitines of chain length 14–20 carbon atoms, alkylsulfobetaines above 14 carbon atoms, and phosphatidylcholines with acyl groups having above 10 carbon atoms is relatively insensitive to chain length. We suggest that this is caused by a balance between increasing hydrocarbon volume promoting the H_{II} phase through decreased intrinsic radius of curvature and greater relief of hydrocarbon packing constraints vs greater intermolecular interactions favoring the more condensed L_α phase. This latter effect is more important for amphiphiles with large headgroups which can pack more efficiently in the L_α phase. The phosphatidylcholines show a gradual decrease in bilayer stabilization between 10 and 22 carbon atoms. This effect is greater in the two-chain amphiphiles than in the single-chain amphiphiles which have only half as much hydrocarbon for the same chain length. Acylcarnitines above a chain length of 20 exhibit a decrease in bilayer stabilization.

The phospholipids of biological membranes are normally organized as bilayers (L_α phase). However, it has long been recognized that some lipid components of these membranes spontaneously form an inverted hexagonal phase structure (H_{II} phase) composed of cylindrical micelles with an aqueous core (Cullis & De Kruijff, 1978; Dekker et al., 1983). It is still controversial as to whether structures resembling the H_{II} phase are intermediates in membrane function. However, it is clear that membrane function can be modulated by substances which affect L_α–H_{II} interconversions. For example, membrane fusion is inhibited by substances which favor the L_α phase. A bilayer stabilizer may favor the L_α phase either by lowering the energy of the L_α phase or by raising the energy of the H_{II} phase. A number of these bilayer-stabilizing compounds have been shown to have antiviral activity including carbobenzoxy-D-Phe-L-Phe-Gly (Richardson et al., 1980; Epand, 1986), cyclosporin A (Gui et al., 1983; McKenzie et al., 1987), tromantadine (Lieb & May, 1972; Cheetham & Epand, 1987), carbobenzoxy-L-Ser-L-Leu-amide (Epand et al., 1987), carbobenzoxy-D-Phe-L-Leu-Gly-D-Phe-D-Leu-D-Leu, and carbobenzoxy-D-Phe-L-Leu-Gly-D-Phe-D-Leu-D-Leu-Gly (Lobl et al., 1988). In addition, the activity of several membrane-bound enzymes is altered by substances which affect L_α–H_{II} interconversion. For example, protein kinase C is inhibited by uncharged and zwitterionic compounds which are bilayer stabilizers (Epand, 1987; Epand et al., 1988, 1989).

To further understand the factors which affect the L_α–H_{II} transition, we have undertaken a systematic study of several series of amphiphiles of varying chain length. The L_α–H_{II} transition temperature of phosphatidylethanolamines is particularly susceptible to the presence of low concentrations of certain amphiphiles (Epand, 1985). The series of amphiphiles we use in the present study are alkyltrimethylammonium

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bromides, alkyl sulfates, alkylsulfobetaines, acylcarnitines, and diacylphosphatidylcholines. Certain diacylphosphatidylcholines have previously been shown to prevent H_{II} phase formation (Tate & Gruner, 1987; Epand & Bottega, 1988). The other compounds are all detergents. Other detergents have been shown to inhibit H_{II} phase formation (Madden & Cullis, 1982). However, this is the first study which explains the effect of amphiphile chain length on the L_α–H_{II} phase transition of phosphatidylethanolamines.

EXPERIMENTAL PROCEDURES

Materials

Dielaidoylphosphatidylethanolamine (DEPE) was purchased from Avanti Polar Lipids (Birmingham, AL). Its purity was verified by TLC and DSC analysis. The acylcarnitines from C12 to C18, the alkyltrimethylammonium bromides from C12 to C16, and the phosphatidylcholines were purchased from Sigma Chemical Co., St. Louis, MO. The C8 and C14 alkyl sulfates were from Aldrich Chemical Co., Milwaukee, WI. The C12 alkyl sulfate was from Bio-Rad, Richmond, CA, and the C16, C18, and C20 alkyl sulfates were from Research Plus, Inc., Bayonne, NJ. The C8–C16 alkylsulfobetaines (Zwittergents) were from Calbiochem, La Jolla, CA. The C18 alkylsulfobentaine and the C20 acylcarnitine were from Serva Feinbiochemica, Heidelberg.

Long-chain acylcarnitines were prepared by a procedure similar to that used for palmitoylcarnitine (Brendel & Bressler, 1969). DL-Carnitine hydrochloride (Sigma) was converted to the perchlorate salt with $AgClO_4$. Acylation of carnitine was carried out with behenoyl chloride (C22) or with lignoceryl chloride (C24), which were purchased from Nu Chek Prep, Elysian, MN. The acylation was carried out for 4 days in acetonitrile at 40 °C with constant stirring. The product was precipitated out with a mixture of ether and hexane and dried. Purity was ascertained by the presence of a single spot in TLC developed with chloroform/methanol/acetic acid/water (40/20/1/1 v/v) as well as positive-ion fast atomic bombardment mass spectrometry. The behenoylcarnitine chloride (C22) had a melting point of 105–120 °C and an ^{1}H NMR spectrum corresponding to a long-chain acylcarnitine. It showed a major peak in mass spectrometry of 484 (theory for behenoylcarnitine cation, 484). The lignocerylcarnitine chloride (C24) had a melting point of 94–98 °C and an ^{1}H NMR spectrum corresponding to a long-chain acylcarnitine. The major peak in mass spectrometry was 512.5, corresponding to the mass of the lignocerylcarnitine cation.

The procedure for the sulfation of decanol was adapted from method B of Sobel and Spoerri (1941). Briefly, 1–2, g of dodecanol was added to 20 mL of benzene and an equimolar amount of pyridinesulfur trioxide and 1 mL each of acetic anhydride and pyridine. The reaction mixture was maintained at 50–60 °C for 2–3 h. The mixture was then cooled to room temperature, 80 mL of petroleum ether added, and the mixture placed in the freezer for 1 h. A solid separated, and the supernate was discarded. The solid was dissolved in 10–20 mL of chloroform and undissolved material discarded. Four volumes of petroleum ether were added to the chloroform solution, and the solid product was separated. This procedure was repeated. The dried product was suspended in water at a concentration of 5% and an equal volume of 10% NaCl was added and the pH adjusted to 7. The solid product was recovered, dried, and recrystallized from methanol. The product had a melting point above 250 °C with decomposition. Fast atomic bombardment spectroscopy gave a molecular mass of 237 (decyl sulfate anion = 237). The product gave one spot on TLC and an element analysis of C, 41.9%; H, 6.9%; S, 10.7% (theory: C, 46.2%; H, 8.1%; S, 12.3%).

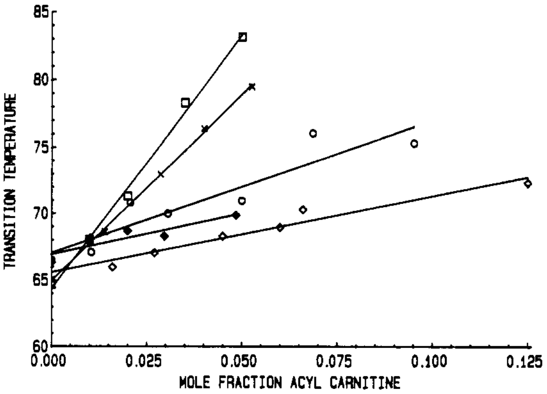

FIGURE 1: Dependence of the L_α–H_{II} transition temperature of DEPE on the mole fraction of added acylcarnitine. C12 (◇), C16 (×), C20 (□), C22 (♦), C24 (○).

Methods

Differential Scanning Calorimetry (DSC). The phosphatidylethanolamine and any added amphiphile were codissolved in a solution in chloroform/methanol (2/1 v/v). The solvent was evaporated with a stream of nitrogen so as to deposit the solutes as a film on the wall of a glass test tube. Final traces of solvent were removed in a vacuum desiccator at 40 °C for 1 h. The lipid films were suspended in 20 mM Pipes, 1 mM EDTA, and 150 mM NaCl with 0.002% NaN_3 at pH 7.4 by vortexing at 45 °C for 30 s. The final lipid concentration was 5 mg/mL. The lipid suspensions were degassed under vacuum before being loaded into an MC-2 high-sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A scan rate of 39 K/h was employed. Duplicate heating runs and duplicate samples were often run to ensure reproducibility of the observations. In no case did we observe major alterations in thermotropic properties as a result of differences in samples or sample history. The enthalpy of the transitions of DEPE shows a variability of ±25% because this lipid does not form a well-dispersed homogeneous suspension. The enthalpy is calculated per mole of phosphatidylethanolamine. In no case was the mole fraction of additive greater than 0.1. Any contribution that the additive itself makes to the observed enthalpy is likely to be within the experimental error of the measurement. The temperature of the main transition is reported as the temperature of maximal excess heat capacity. The bilayer to hexagonal phase transition was fitted to a single van't Hoff component and the transition temperature reported as that for the fitted curve.

RESULTS

The primary objective of this study was to determine how the chain length of membrane additives can change the L_α–H_{II} transition temperature of DEPE. The phase transition properties of a series of five to eight samples of DEPE with each species of amphiphile were measured by DSC. The mole fraction of added amphiphile was varied between 0 and usually 0.1. The temperature of the L_α–H_{II} transition was plotted as a function of the mole fraction of added amphiphile present in the mixture. These plots were linear over the range of amphiphile concentrations used. The data for some of the acylcarnitines are presented in Figure 1 as an example. The effect of the amphiphiles on the L_α–H_{II} transition temperature is summarized as the slope of such plots. The dependence of the slope on amphiphile chain length is shown in Figure 2 for the several classes of compounds studied.

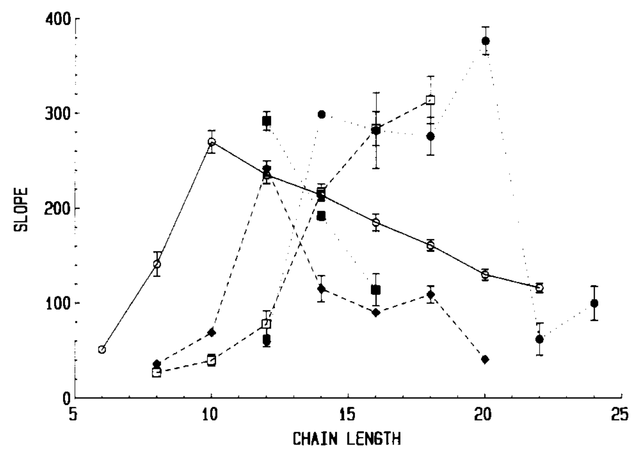

FIGURE 2: Dependence of the slope (degrees centigrade per mole fraction of additive) of the linear regression line of the L_α–H_{II} transition temperature of DEPE vs the mole fraction of additive. Error bars for the slope of this line are included, but generally fall within the points. Each point represents DSC scans on five to eight different samples. Acylcarnitines (●···●), alkyl sulfates (♦---♦), alkyltrimethylammonium bromides (■···■), alkylsulfobetaines (□---□), phosphatidylcholines (○—○).

The L_α–H_{II} transition becomes broader with an increasing mole fraction of additive, resulting in about a 2–5-fold decrease in the van't Hoff enthalpy over the range of additive concentrations used. The calorimetric enthalpy of the L_α–H_{II} transition is 450 cal/mol of DEPE at 65.6 °C for the pure lipid. The ΔH does not vary in a substantial or systematic manner for any of the samples with added amphiphile except for several of the samples with acylcarnitines. The acylcarnitines of chain length 14 carbons or greater lowered the transition enthalpy to about 200 cal/mol of DEPE at an acylcarnitine mole fraction of 0.1.

The L_β–L_α transition temperature is generally 10-fold less sensitive to the presence of additive than the L_α–H_{II} transition temperature (Epand, 1985). Additives generally decrease the L_β–L_α transition temperature. This is also true for the amphiphiles studied here with some exceptions. Amphiphiles which did not cause a shift in the L_β–L_α transition temperature are the C22 and C24 acylcarnitines, the C16 and C18 alkyl sulfates, the C18 sulfobetaine, and the C16 phosphatidylcholine. Amphiphiles which raise the L_β–L_α transition temperature by about one-tenth the amount they raise the L_α–H_{II} transition temperature are the C20 alkyl sulfate and the C18, C20, and C22 chain length phosphatidylcholines. The enthalpy of the L_β–L_α transition is 6 kcal/mol at 37.2 °C and is invariant with added amphiphile.

DISCUSSION

The chain length dependence of the effects of amphiphiles on the L_α–H_{II} transition temperature can be explained by three factors: increased hydrocarbon bulk with longer chain lengths, greater hydrophobic interactions in the low-temperature phase, and changes in partitioning of the added amphiphile between water and DEPE.

The first of these factors has long been recognized. It was first described as a change from a cylindrical to a cone-"shaped" molecule favoring the H_{II} phase (Israelachvilli et al., 1980). Recently, Gruner (1985) has attempted to develop a more accurate and quantitative theory. Phase preference is described as a competition between the intrinsic radius of curvature of a lipid monolayer and hydrocarbon packing constraints. The intrinsic radius of curvature describes the tendency of a lipid monolayer to curl from a planar flat surface. The smaller the intrinsic radius of curvature, the greater the tendency to form the hexagonal phase. The intrinsic radius of is curvature is determined by molecular interactions in both the headgroup and hydrocarbon regions of the membrane. It is not currently possible to calculate this parameter, and it must be determined empirically. As the intrinsic radius of curvature gets larger, formation of the hexagonal phase is prevented by hydrocarbon packing constraints. This hydrocarbon packing problem occurs in the hexagonal phase as a result of the voids between the cylinders which must be filled by intercalation of the acyl chains. The larger the hexagonal phase tube diameter, the larger are the voids which must be filled and hence a greater energy required to overcome these packing constraints. The two factors, i.e., intrinsic radius of curvature and hydrocarbon packing constraints, can be distinguished on the basis of cylinder diameter (Tate & Gruner, 1987). If hydrocarbon packing constraints are increased, then the cylinder diameter will decrease, while if the intrinsic radius of curvature increases, then the cylinder diameter will increase. Short-chain amphiphiles would be expected to stabilize the bilayer phase by both increasing the hydrocarbon packing constraints because their chain lengths are shorter than those of the host phospholipid and also increasing the intrinsic radius of curvature because they have large and hydrated headgroups and relatively little hydrocarbon. Bilayer stabilization caused by either of these factors will decrease with increasing amphiphile chain length. Although the longer amphiphiles may not relieve hydrocarbon packing constraints relative to the pure phospholipid, the hydrocarbon packing problem in the H_{II} phase will become less unfavorable than it is with the shorter amphiphiles. We have attempted to distinguish these two factors by measuring hexagonal phase spacings with X-ray diffraction (A. Sen, unpublished observations). Samples of DEPE containing 5 mol % added sulfate, acylcarnitine, or sulfobetaine of varying chain length were studied in the L_α phase and in the H_{II} phase at a temperature 5 °C above the $L_\alpha \rightarrow H_{II}$ transition temperature. The spacings are precise to ±0.25 Å. There is no change in the bilayer spacings for any of the samples compared with the DEPE spacing of 68.2 Å. Half of the samples showed no differences with DEPE in the H_{II} phase spacings of 65.2 Å. The greatest difference found was only 1.8 Å. Some amphiphiles increased the H_{II} spacings while others decreased it. The observed differences were small and did not vary in a consistent manner with either amphiphile chain length or class of amphiphile. The dependence of the H_{II} spacings on amphiphile concentration and temperature was not studied. Since the differences were small and did not add to our understanding of the amphiphile chain length dependence, the study was not pursued further at this time. We therefore do not distinguish between effects caused by hydrocarbon packing constraints vs intrinsic radius of curvature. We wish to point out, however, that both factors are expected to vary in the same direction in going from shorter to longer chain length amphiphiles.

The second factor is simply a restatement of the observation that longer chain amphiphiles have higher melting or phase transition temperatures than shorter chain homologues. This is a result of increased intermolecular interactions with longer chains. Since the density of the low-temperature phase, and hence intermolecular interactions, is higher than in the high-temperature phase, the low-temperature phase becomes more stable with longer chain length. This phenomenon is illustrated in Figure 3. As a result, the L_α–H_{II} transition temperature increases with increasing chain length. This is opposite to the effects of hydrocarbon packing constraints or intrinsic radius of curvature.

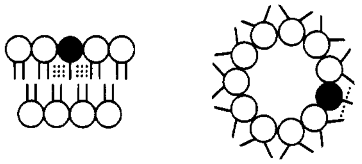

FIGURE 3: Schematic representation of the cross section of the bilayer phase (left) and a H_{II} phase tube. The added amphiphile is distinguished by having a headgroup with a solid circle. Some of the intermolecular interactions between the added amphiphile and the phospholipid are shown with dashed lines. Note that in the H_{II} phase, because of the lower density and less regular packing, these interactions are weaker.

The final consideration is partitioning of the added amphiphile. There are two aspects of partitioning. These are partitioning of the amphiphile between aqueous and membrane phases and partitioning or lateral phase separation between the added amphiphile and DEPE within the membrane phase. Aqueous partitioning will be important for small chain length amphiphiles. It explains why all of the amphiphiles have a decreased effect on the slope as one goes to short chain lengths (Figure 2). This was not observed for the alkyltrimethylammonium bromides over the range of chain lengths studied, but clearly one would have to come to a point where these amphiphiles would partition only into water. Tetramethylammonium bromide has no effect on the L_α–H_{II} transition temperature up to a mole fraction of 0.2 (data not shown). Lateral phase separation is more difficult to evaluate quantitatively. The L_α–H_{II} transition can be observed by DSC only over a limited range of concentrations of added amphiphile before the transition becomes too broad. Hence, only a limited region of the phase diagram can be constructed. However, all of the amphiphiles tested decreased the van't Hoff enthalpy to similar extents at similar mole fractions, suggesting that the amphiphile was mixing with the DEPE.

There is a marked contrast between the behavior of alkyl sulfates vs acylcarnitines (Figure 2). In the former case, increasing the chain length above 12 decreases the bilayer-stabilizing effect as a result of increasing the amount of hydrocarbon. In contrast, the slope for acylcarnitines is insensitive to chain length between chain lengths of 14 and 20. It has also been observed that the bilayer to nonbilayer phase transition temperature of a series of glycosyldiacylglycerols is insensitive to chain length between C14 and C18 (Mannock et al., 1988). Thus, the effects of longer amphiphile chain length cannot be predicted solely on the basis of the expected changes in the intrinsic curvature and hydrocarbon packing in the H_{II} phase resulting from the increased amount of hydrocarbon. We ascribe this insensitivity to increasing chain length as a balance between opposing effects. Increased chain length causes increased monolayer curvature and relieves H_{II} phase hydrocarbon packing constraints relative to shorter chain amphiphiles resulting in a smaller slope, while increased intermolecular interactions in the L_α phase favors a larger slope with increasing chain length. This latter effect is more pronounced for membrane additives with a particular balance between hydrophobic and hydrophilic groups. Single-chain amphiphiles with relatively large polar headgroups, such as acylcarnitines, can pack better in the L_α phase than in the H_{II} phase. The slopes of alkylsulfobetaines also appear insensitive to chain length (C14–C18), although the range of compounds used is more limited for this series. Single-chain amphiphiles with small headgroups, such as the alkyl sulfates, may pack equally well in the L_α and H_{II} phase. Hence, intermolecular interactions, which increase with increasing chain length, will not greatly favor one phase or another. Thus, effects of hydrocarbon packing constraints and the intrinsic radius of curvature will predominate, making longer chain sulfates less bilayer-stabilizing. The single charged amphiphiles will also have a bilayer-stabilizing effect because of electrostatic repulsion. This repulsion will be greater in the H_{II} phase where the headgroups are more tightly packed. The case of the alkyltrimethylammonium bromides appears similar. The phosphatidylcholine series is intermediate. The slopes of the phosphatidylcholines decrease with increasing chain length but not as fast as the singly charged amphiphiles. The phosphatidylcholine headgroup is large, but for a given chain length, there is twice as much hydrocarbon compared with the single-chain amphiphiles. However, the hydrocarbon effect is less than that of a single-chain amphiphile of twice the length, since the latter will be more effective in relieving H_{II} phase hydrocarbon packing constraints because of its greater penetration into the membrane. The longest chain length phosphatidylcholines are in the gel state around the L_α–H_{II} transition temperature of DEPE (Keough et al., 1987). Nevertheless, there is little change in slope in going from below 18 to 20 or 22 carbon atom acyl chain phosphatidylcholines. This again indicates that the additives are mixing with DEPE since domains of the L_β or L_α phase of phosphatidylcholine would have markedly different effects on the transitions of DEPE.

The acylcarnitines exhibit a marked change in slope between 20 and 22 carbon atoms. This is close to the length of DEPE. Elaidic acid is 18 carbons with a trans double bond between C9 and C10. We suggest that when the additive is longer than the host phospholipid then the additional hydrocarbon does not add to intermolecular interactions in the L_α phase but does increase monolayer curvature and relieves H_{II} phase hydrocarbon packing constraints even relative to the pure DEPE.

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Registry No. DEPE, 16777-83-6; C12 acylcarnitine, 25518-54-1; C6 acylcarnitine, 2364-67-2; C20 acylcarnitine, 80600-92-6; C22 acylcarnitine, 85110-52-7; C24 acylcarnitine, 123359-35-3.

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A New Method for Extraction of Iron–Molybdenum Cofactor (FeMoco) from Nitrogenase Adsorbed to DEAE-cellulose. 1. Effects of Anions, Cations, and Preextraction Treatments[†]

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ABSTRACT: A convenient and rapid method of obtaining the cofactor of nitrogenase (FeMoco) with a low and apparently limiting Fe/Mo ratio has been developed. FeMoco can be extracted from the MoFe protein bound to DEAE-cellulose. The cofactor is eluted in either *N*-methylformamide (NMF), *N*,*N*-dimethylformamide (DMF), or mixtures of these solvents by use of salts such as Et_4NBr, Bu_4NBr, Ph_4PCl, and Ph_4AsCl. The method is simple, is rapid (45 min), yields concentrated cofactor, and, unlike the original method [Shah, V. K., & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249–3253] which requires anaerobic centrifugation, is easily scaled up. Furthermore, it gives yields of cofactor in excess of 70%. Its disadvantages are a high Fe:Mo ratio when DMF is the extracting solvent and a high salt concentration in the resultant FeMoco solution. These disadvantages are easily overcome by removing excess Fe by pretreating the cofactor with bipyridyl while still on the column. This gives Fe:Mo ratios of (6 ± 1):1 (11 trials) with specific activities ranging from 170 to 220 nmol of C_2H_4/[min·(nmol of Mo)]. Chromatography on Sephadex LH-20 removes ca. 99% of the excess salt. The adsorption of MoFe protein to DEAE-cellulose seems to facilitate denaturation by organic solvents so that pretreatment of the protein with acid, used in earlier methods, is unnecessary. There is an apparent dependence on the charge density of the anion employed for elution of FeMoco bound to DEAE-cellulose, such that $Cl^- > Br^- \gg I^-, PF_6^-$ is the order of effectiveness of the Bu_4N^+ salts of these anions. The extraction of cofactor in DMF with a comparable specific activity to that obtained in NMF proves that the two amides are equally good solvents for the cofactor and coordination of deprotonated solvent may not be *necessary* for extraction.

Nitrogenase consists of two proteins, the Fe protein and the MoFe protein [for a review, see Orme-Johnson (1985)]. The Fe protein is an $\alpha_2\beta_2$ tetramer with a metal content of Mo_2Fe_{30-32} (Davis & Orme-Johnson, 1976). It contains an extractable MoFe–sulfur cluster (Shah & Brill, 1977) which is believed to be the active site for dinitrogen reduction. For example, the altered substrate reduction properties of the MoFe protein from *Klebsiella pneumoniae nifV* mutants are transferred to apoprotein via the cofactor extracted from the mutant protein (Hawkes et al., 1984). The product of the *nifV* gene appears to be required for the production of homocitrate, whose presence is needed for the synthesis of wild-type cofactor (Hoover et al., 1987). Mössbauer studies have shown that two distinct classes of iron centers (designated P and M centers) are present in MoFe protein (Münck et al., 1975; Zimmermann et al., 1978; McLean et al., 1987). The P clusters consist of four $[Fe_4S_4]$ centers (Zimmermann et al., 1978) while the two M centers each correspond to FeMoco (Huynh et al., 1980).

FeMoco can be extracted into NMF[1] from citric acid precipitated MoFe protein with an efficiency variously reported to be between 45 and 90% (Shah & Brill, 1977; Yang et al., 1982; Walters et al., 1986). The partial formula of the resultant FeMoco is $MoFe_{6-8}S_{8-9}$ (Nelson et al., 1983). The cluster extracted in NMF can reconstitute MoFe protein ac-

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[1] Abbreviations: bpy, 2,2′-bipyridine; DEAE-cellulose, (diethylaminoethyl)cellulose; DMF, *N*,*N*-dimethylformamide; EPR, electron paramagnetic resonance; NMF, *N*-methylformamide; TEAE-cellulose, (triethylaminoethyl)cellulose; Tris, tris(hydroxymethyl)aminomethane.

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