than the reactivity shown by the free flavins FAD and FMN themselves. Other flavoproteins show low reactivity with sulfite. It was therefore interesting that, unlike lipoamide dehydrogenase,[23] flavopapains **1C**, **2C**, and **3C** form reversible adducts with sulfite. The K_d constants for the formation of sulfite adducts of **1A**, **1C**, **2A**, and **2C** are 4, 15, 5, and 16 mM, respectively (pH 7.5, 0.02 M Tris, 0.08 M KBr, 0.1 mM EDTA). The enhanced stability of sulfite adducts of the flavin-dependent oxidase enzymes has been correlated with protein stabilization of a flavin radical. Given the similarity in magnitude of the K_d values, it appears that the papain environment has little influence on the addition of sulfite to the N-5 position.

Conclusions

The results of this work provide additional evidence that chemical modification of existing proteins is certainly a viable method for producing new catalysts. In particular, a catalyst of novel structure has been constructed that mediates the efficient oxidation of dithiols to disulfides. The better understanding of the diverse catalytic efficiency exhibited here and in previous studies remains a worthwhile goal. It appears that the different substitution pattern in **1C**, **2C**, and **3C** has created active sites of different shape, and for two classes of substrates, the active site of **1C** appears to allow the closest approach of substrate to flavin in a manner that is along a productive reaction pathway. The versatility of the semisynthetic flavopapain catalysts is shown by their ability to oxidize dithiols and dihydronicotinamides. Future work will continue to focus on establishing general rules for optimizing the design of semisynthetic enzymes.

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Registry No. 1A, 79127-38-1; **1B**, 79127-42-7; **2A**, 63147-98-8; **2B**, 68973-54-6; **3B**, 101916-86-3; *d,l*-$HS(CH_2)_2CH(SH)(CH_2)_4CONH_2$, 4265-09-2; *d,l*-$HS(CH_2)_2CH(SH)(CH_2)_4CO_2H$, 7516-48-5.

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Praseodymium(III) Transport across Phospholipid Vesicles in the Joint Presence of an Ionophore and a Fatty Acid†

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Abstract: Accelerated transport of Pr^{3+} ions across phosphatidylcholine vesicles, as measured by ^{31}P NMR, occurs in the joint presence of an ionophore (X-537A) and a fatty acid, itself totally incapable of Pr^{3+} transport. Several mechanisms are considered in turn, and they are ruled out by appropriate experiments. The explanation for the observed synergism is coupling of Pr^{3+} influx with H^+ (and Na^+) efflux, in an "antiport" mechanism. The fatty acid present helps in this efflux: either because of its pK_a difference with X-537A and/or because it serves as an uncoupler.

A remarkable synergism occurs in the transport of Pr^{3+} across phosphatidylcholine vesicles: adjunction to lasalocid A (X-537A), a bona fide ionophore (A), of another ionophore (B), such as etheromycin or monensin, boosts the rate of transport.[1] These initial observations were later generalized to include quite a number of paired ionophores of the polyether antibiotic type.[2] Moreover, a similar synergism occurs when, instead of a second ionophore, a synthetic crown ether is incorporated in the lipid bilayer together with X-537A.[3]

All these findings applied to accelerated transport by a 2:1 hybrid complex, in which the Pr^{3+} cation is coordinated to both ionophores A and B. All these findings applied to the influx of Pr^{3+} from the outside of the lipid bilayer to the inside of the vesicles. A common structural feature of all the species B that displayed, jointly with an ionophore A, synergistic transport of Pr^{3+} was the presence of a carboxylic group. Therefore, our working hypothesis was that, because of the necessary coupling between Pr^{3+} influx and H^+ efflux for maintenance of electroneutrality, the presence of a ΔpK_a between both acidic groups in the hybrid (Pr^{3+}, A+B) complex could facilitate back-transport of protons and thus render Pr^{3+} transport faster than in the "pure" (Pr^{3+}, A_2) or (Pr^{3+}, B_2) complexes.[2] We test here this working hypothesis by studying Pr^{3+} transport in the joint presence of an ionophore (X-537A) and a fatty acid, which in itself is totally incapable of Pr^{3+} transport. We find indeed that the cation inward rate is markedly enhanced by the presence of the fatty acid.

Materials and Methods

Suspensions of vesicles were prepared as previously described[2] from a modification of a published procedure.[4] The phospholipidic content was typically 30 mM, and the pH of the aqueous solution was adjusted with NaOH. Methanolic solutions of lasalocid A (Aldrich) and of the various fatty acids (Sigma or Aldrich) were added to the vesicle suspensions and incubated for at least 15 min at the temperatures of the various experiments. Palmitic acid ^{13}C-labeled on the carboxyl carbon was incorporated for some of the experiments into the bilayer by sonication.

The acidity constants K_a have been determined by potentiometry,[5] with an ion analyzer, Orion Model 701A, in methanol-water mixtures (80:20, v/v), a medium that simulates very well the interface between a membrane and water.[6] Ion-exchange chromatography (Dowex 50W ion

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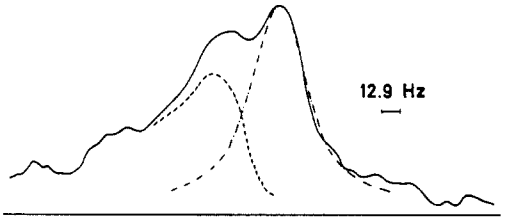

Figure 1. Example of deconvolution of an experimental line shape (—): subtraction of the high-field Lorentzian component (···) due to vesicles devoid of internal Pr^{3+}.

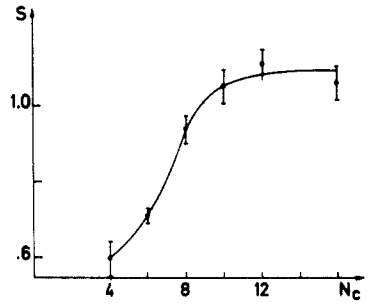

Figure 2. Influence of the chain length of fatty acids, as measured by the number of carbon atoms N_c, on carrier-mediated (X-537A) transport of Pr^{3+} as measured by the parameter S (Hz min^{-1}). The determining factor is incorporation of the fatty acid in the lipidic bilayer (see text).

exchanger) provided lasalocid A under its acidic form. The transport rates are determined as in our earlier papers[1,2] by the NMR technique pioneered by the Bystrov group.[7] The ^{31}P NMR spectrum of the suspension of the vesicles, in the absence of the paramagnetic praseodymium(III) ions, shows but one peak. The presence of Pr^{3+} in the external compartment shifts downfield the ^{31}P resonance of the outer polar head groups. Penetration of the paramagnetic cations inside the vesicles induces also the downfield shift of the inner phosphate signal, whose shift rate thus monitors Pr^{3+} transport.

The ^{31}P NMR spectra have been obtained either with a Bruker WP-80 or with a Bruker AM-300 WB spectrometer, with nominal frequencies of 32.39 and 121.44 MHz. In the latter case, the dispersion of the chemical shifts makes it possible to distinguish, during the early part of each kinetic run, the vesicles without any paramagnetic Pr^{3+} ion inside from those having at least one such ion. Therefore, we could then determine the ^{31}P chemical shift of the "inner" signal after subtraction of a Lorentzian line corresponding to those vesicles devoid of Pr^{3+}. A typical example of this deconvolution procedure is shown in Figure 1.

The longitudinal relaxation times T_1 have been measured with the AM-300 WB apparatus by using the $(T-180°—t-90°)_n$ pulse sequence. Thirteen t values have been used to calculate the T_1's by fitting the experimental points to an exponential curve. The ^{13}C NMR spectra have been determined on the same high-field spectrometer operating at 75.43 MHz.

Results and Discussion

Analysis of the kinetic data shows confirmity to a diffusive carrier mechanism.[8] According to this valuable kinetic model, the progressive broadening of the inner resonance is consistent with an intermediate rate of mediator exchange between vesicles, relative to the rate of translocation.

As we expected, the transport rate is increased significantly when fatty acids are present. We have investigated the effect of varying the length of the alkyl chain R of the saturated fatty acids RCOOH (Figure 2).

A sigmoidal change is observed, plateauing to a maximum when the chain has 10 carbon atoms or more. The simplest interpretation involves the relative solubilities of these fatty acids in the aqueous and in the lipid phases: while butyric, *n*-caproic, and caprylic acids are soluble in water [miscible; 1.08 g/100 g and 0.068 g/100 g, respectively (Merck Index)], starting with *n*-capric acid, which is practically insoluble in water, the higher homologues are lipophilic and will dissolve more readily in the lipidic bilayer.

Similarly, this can be also expressed from the Hansch partition coefficients.[9] Their logarithms are −3.2, −2.2, −1.2, −0.2, and +0.8 for the C_4, C_6, C_8, C_{10}, and C_{12} acids, respectively, paralleling the behavior observed here (Figure 2).

To determine why incorporation of a fatty acid into a phosphatidylcholine vesicle increases the rate of influx of Pr^{3+} ions transported by X-537A from the oustide to the inside of the vesicle, we first checked that the observed effect is not due to major disruption of the membrane structure. A first possibility would be for insertion of fatty acid molecules into the lipidic membrane to increase the density of negatively charged groups at the surface of the vesicle: thus, more cations would be attracted, condensed, or bound into the electrical double layer, and the transport rate would be increased accordingly. But we could rule out such an explanation, at least as the predominant factor; in the Pr^{3+} concentration range investigated, the downfield shift of the outer phosphate signal (see Materials and Methods) is proportional to the mole fraction of bound Pr^{3+}. Addition of lauric acid (lauric acid/phospholipid = 0.01) induces an extra shift of 5%. This would translate into a 5% increase in the transport rate, which is negligible by comparison to the observed increase of 250%.

A second possibility is for insertion of fatty acids into the lipidic membrane to disrupt its structure to such an extent that Pr^{3+} transport will be boosted. We have investigated this factor by determining the ^1H longitudinal relaxation times of the lipid bilayer as a function of the fatty acid concentration (Table I). Proton relaxation in membranes is dominated by dipolar interactions that are partially averaged by fast internal motions of the membrane lipids. Appropriate models describing the dynamics of the phospholipid chains have been reported in the literature.[10-12] The slow isotropic tumbling of the vesicles and the lateral diffusion of the lipids are also responsible for the partial averaging of the dipolar interactions.[13,14] A partial leveling of the proton relaxation times comes from a slow spin-diffusion process within the bilayer.[15] The insertion of fatty acids in the lipidic phase induces perturbations in the mobility of the membrane,[16] and it should be reflected by proton relaxation times.

The signal assingments have been taken from the literature.[17] No significant change in the mobility due to the presence of the fatty acid is displayed by the results. The absence of a significant effect is due to the weak content of free fatty acid, ca. 1% instead of the 20% in the study by Bloom et al.[16]

A third possibility is a chemical change in the composition of the membrane lipids induced by the presence of free fatty acid:

Table I. Proton Longitudinal Relaxation Times T_1 (s) of Phosphatidylcholine Vesicles (30 mM) with and without Lauric Acid

		concn lauric acid, mM		
assgnt	δ	0	0.221	0.442
CH=CH, CH-OCO	5.35	0.40 (±0.02)	0.44 (±0.02)	0.47 (±−0.001)
CH_2OP, CH_2OCO	4.34	0.35 (±0.01)	0.35 (±0.01)	0.37 (±0.01)
CH_2N^+	3.74	0.31 (±0.01)	0.31 (±0.01)	0.31 (+0.01)
$(CH_3)_3N^+$	3.29	0.36 (±0.01)	0.35 (±0.01)	0.39 (±0.01)
CH_2CO	2.41	0.35 (±0.01)	0.33 (±0.01)	0.35 (±0.01)
CH_2C=	2.08	0.40 (±0.01)	0.41 (±0.01)	0.43 (±0.01)
$(CH_2)_n$	1.31	0.44 (±0.01)	0.44 (±0.01)	0.45 (±0.01)
CH_3	0.93	0.63 (±0.01)	0.64 (±0.02)	0.69 (±0.01)

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Table II. pH Dependence of the Pr^{3+} Transport Rate[a]

pH	slope (±σ)	ρ	concn lauric acid, mM
6.21	1.00 (±0.02)	0.992	0
	1.61 (±0.08)	0.991	0.29
6.40	1.31 (±0.03)	0.997	0
	1.56 (±0.05)	0.991	0.07
	2.20 (±0.08)	0.995	0.15
	3.66 (±0.28)	0.987	0.29
	7.20 (±0.40)	0.994	0.44
	8.60 (±0.37)	0.995	0.58
6.51	1.59 (±0.09)	0.988	0
	8.06 (±0.50)	0.993	0.29
6.62	1.70 (±0.07)	0.994	0
	6.51 (±0.25)	0.996	0.15

[a] Key: $[Pr^{3+}]$ = 1.03 mM; [X-537A] = 0.11 mM; ρ = linear correlation coefficient (8–10 points); T = 305 K.

addition of RCOOH molecules might cause hydrolysis of the ester bonds in the phosphatidylcholines with release of complex higher fatty acids, some of which might act as Pr^{3+} carriers. But this would cause a comparable increase in the cation-transport rate with all the acids studied, contrary to what is observed (Figure 2). In addition we could disprove this possibility experimentally. We have examined for this purpose ^{13}C NMR spectra for $^{13}COOH$-enriched palmitic acid incorporated into the vesicular membrane. Acyl transfer between palmitic acid and the phospholipids present would increase the intensity of the ester resonance (δ 174.0) and conversely deplete the free carboxylic groups (δ 175.5). Even after 1 week, the intensity ratio of these two signals in fact remained unchanged.

In order to ascertain the mechanism for the observed synergism in the presence of fatty acids ($N_c > 10$, Figure 2), we elected lauric acid (C_{12}) as a representative case for further study. First, we checked that it does not belong in the class of lipidic compounds such as cardiolipin for which ionophoretic properties have been claimed:[18,19] under the conditions of our experiments, lauric acid does not transport Pr^{3+}. *Yet, in association with lasalocid A, lauric acid gives rise to an enhanced transport rate* (Figure 2).

This is a property of the free acid. By contrast to lauric acid, the methyl laurate ester does not increase translocation of the paramagnetic cation mediated by lasalocid A: presence of the carboxylic or the carboxylate group is necessary to enhance the influx rate of Pr^{3+}; the same is true with higher fatty acids, which are known to be extensively incorporated into the bilayer.[16,20] Furthermore, the pH dependence of the observed enhancement implies, as expected, intervention of the carboxylate group (Table II).

Added insight into the mechanism of this synergism is obtained from the dependence of the cation inward rate upon the concentrations of lasalocid A and lauric acid. At pH 6.5, the rate is second order in lasalocid A [correlation coefficient 0.991 (five points) (Table III)] exactly as had been reported for the ionophore alone,[21,1] and the transport rate is first order with respect to lauric acid: correlation coefficients are 0.991 (five points) at pH 6.0 (Table III) and 0.988 (four points) at pH 6.4 (Table II).

If penetration of the complex into the membrane is rate determining,[22] the role of lauric acid could be that of a shield around the Pr^{3+}–(lasalocid A)$_2$ complex to provide it with better hydrophobicity. Indeed, in liquid–liquid extraction by carboxylic crown ethers, 2:1 stoichiometries (crown ether/univalent or divalent cation) have been proposed for the cationic complex involved in the metal extraction:[23] the second complexant molecule serves

Table III. Pr^{3+} Transport Rate vs. Lauric Acid or Lasalocid A Concentration

slope (±σ)	ρ[a]	concn, mM
	Lauric Acid[b]	
0.58 (±0.03)	0.992	0.13
0.73 (±0.03)	0.996	0.20
0.74 (±0.05)	0.994	0.20
1.01 (±0.07)	0.992	0.27
1.85 (±0.09)	0.996	0.40
	X-537A[c]	
0.65 (±0.05)	0.990	0.04
2.47 (±0.01)	0.996	0.08
2.60 (±0.09)	0.994	0.08
6.30 (±0.23)	0.997	0.12
9.28 (±0.55)	0.993	0.16

[a] ρ = linear correlation coefficient (8–10 points). [b] pH 6.0; $[Pr^{3+}]$ = 1.12 mM; [X-537A] = 0.12 mM; T = 305 K. [c] pH 6.5; $[Pr^{3+}]$ = 1.03 mM; [lauric acid] = 0.27 mM; T = 305 K.

as a counterion that can be displaced by picrates for instance. Nevertheless, for the kinetic results analyzed here, it seems unrealistic to assume involvement of a quaternary complex in the slow step. Conversely, if the rate-determining step is diffusion of the Pr^{3+}-transporting complex through the bilayer, a sizable increase in the lipophilicity of this complex might be expected to alter the activation energy of the overall process. We have determined Arrhenius activation energies for the translocation of Pr^{3+} in the presence and in the absence of lauric acid: 92 ± 8 and 88 ± 8 kJ mol^{-1}, respectively. These activation energies, similar in magnitude to those obtained with other ionophores,[24-27] are equal within experimental error!

Cation transport could also be governed by steps other than diffusion of the complex.[28-30] Thus, it should be recalled that Pr^{3+} influx destroys intravesicular electroneutrality. The chloride anion can indeed permeate the vesicles, but at a pH of 6.2 (ΔpH 0), its permeability coefficient is ca. 5×10^{-12} cm/s,[31] i.e., too low. Furthermore, it seems to be due to the transport of HCl.[32] The passive permeability of Na^+ is 1.2×10^{-14} cm/s.[31] True, the sodium efflux can be mediated by the ionophore itself but much less efficiently than for the paramagnetic ion. Therefore, neither Cl^- nor Na^+ fluxes are sufficient to restore internal neutrality under the time scale of our experiments.

By contrast a proton antiport mechanism appears to be operative. In the absence of lauric acid, the ionophore alone provides a pathway for proton countertransport. The proton is translocated as HA_2^- species where A^- is the anionic form of the ionophore. It results from the association of the undissociated ionophore HA and its form A^-.[33,34] Thus, in order to maintain internal neutrality, more lasalocid A molecules are required for the outward flux of protons than for the influx of Pr^{3+}.

The question arises of whether the added lauric acid could provide an extra pathway for proton countertransport. A first line of evidence comes from the increase in the transport rate at an higher pH (Table II). It indicates that the anionic conjugate base is the active ingredient. Such a possibility has been indirectly

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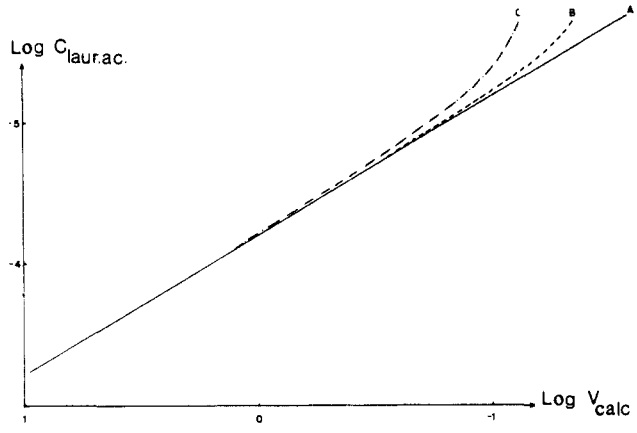


Figure 3. Calculated proton transport rates vs. lauric acid and lasalocid A concentrations, assuming $k_2/k_1 = 0.05$: (A) [X-537A] = 0; (B) [X-537A] = 0.11 mM; (C) [X-537A] = 0.22 mM.

proposed already for myristic and oleic acids.[35] Picric acid, a well-known protonophore,[36,37] also increases Pr^{3+} influx mediated by lasalocid A,[2] which shows that proton efflux is a limiting step for cation influx.

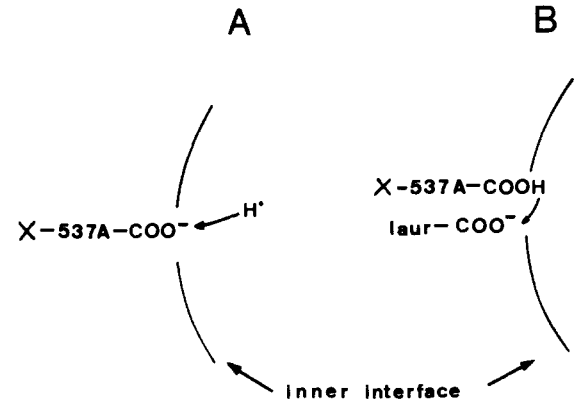
We asked how we could explain the first-order dependence in lauric acid, observed for Pr^{3+} transport, when mediated by the same amount of X-537A. Proton transport can occur by three different processes: passive transport,[38] transport by the lasalocid ionophore A,[33,34] or transport by lauric acid and its conjugate base. In small unilamellar vesicles, the net permeability to protons is 5×10^{-7} cm/s.[38] The uncoupler properties of the ionophore[33,34] render negligible in its presence the passive process. We have estimated a lower limit for the proton efflux due to the presence of a fatty acid as follows: the ^{13}C resonance of $^{13}COOH$-enriched palmitic acid incorporated into the bilayer is pH dependent. If palmitic acid molecules were to crisscross the bilayer at a rate slow on the NMR time scale, and in the presence of an adequate pH gradient across the membrane, one should see two resonances due to the inner and the outer carboxyl groups. At pH 6.2 (ΔpH 0), the carboxyl signal occurs at 176.2 ppm. When the external pH is increased to 8.35 by addition of sodium hydroxide, one single line is observed at 178.7 ppm: on the basis of the chemical shift difference, the flip-flop rate of palmitic acid between the two compartments is at least 2.5×10^{-5} s^{-1}. Taking into account the dimensions of small phosphatidylcholine vesicles,[31] the resulting permeability coefficient[8,39] is 2.5×10^{-2} cm/s, much higher than that for the passive transport of protons (see above). (Note that absence of chloride ions and an internal compartment more acidic than the external are required to avoid passive permeation of protons.[40]) No value for proton translocation in the presence of lasalocid A has been reported. However, the proton transport rate constant mediated by A23187, another powerful ionophore, is 28 s^{-1}.[41]

The proton transport rate coupled to the Pr^{3+} influx can be expressed as a sum of two contributions

$$V_{calcd} = k_1[\text{X-537A}]^2 + k_2[\text{lauric acid}]$$

where k_1 and k_2 are the apparent rate constants for proton fluxes in the presence of lasalocid A and of lauric acid, respectively. Taking into account the above estimates for the rate constants together with the used concentrations in our experiments, the last term is obviously predominant. The second term is about 3 orders of magnitude greater than the first at the highest lauric acid concentration used in the transport experiments (Table II). With such an assumption, the rate V_{calcd} has been plotted vs. the fatty acid and ionophore concentrations (Figure 3). With an ionophore concentration of 0.11 mM, the calculated curve does not deviate significantly from a straight line except at very low lauric acid concentrations. This simulation is in full agreement with our experimental results. As expected, due to the quadratic effect of the lasalocid concentration,[33,34] at higher ionophore levels the curvature occurs at the highest fatty acid concentrations.

Scheme I



The above assumption of a rate law first order in carboxylic acid is consistent with the data in Table II: the kinetic order in H^+, based on these few points, appears as −0.6 with lasalocid alone and between −2 and −3 with added lauric acid. As indicated by the reviewer, these are very reasonable results, as the electroneutral transport by (lasalocid-H–lasalocid$^-$) dimers should have inverse first order in protons,[33] while a lauric acid cycle that achieves electroneutrality should show inverse cube order in protons.

We had earlier postulated that the ΔpK_a intrinsic to the hybrid complex formed between two ionophores might help in "pulling" protons through the water–membrane interface.[2] Obviously, such a process remains possible here. We can visualize such a kinetic effect in the (admittedly improbable, but this would provide the lowest activity energy pathway) situation having the least basic carboxylate group sitting at the interface and in rather close proximity to the most basic carboxylate group, the latter buried more deeply in the lipophilic membrane environment (Scheme I). As soon as a proton would attach itself to the least basic COO^-, its further jumps across the more basic COO^-, its further jumps across the more basic COO^- would become highly probable. Hence, such a mechanism may well facilitate penetration of protons from the inner compartment into the lipid bilayer, and this would accelerate transport, as effected and seen by Pr^{3+}.

One last question that should be addressed is whether a pK_a difference does indeed exist in the lauric acid–lasalocid A system. We have determined the two acidity constants for these carboxylic acids in methanol–water mixtures (80:20, v/v) chosen to mimic the membrane interface.[6] The corresponding pK_a's are 6.98 ± 0.02 and 5.22 ± 0.02 for lauric acid and for X-537A, respectively. The value for the antibiotic agrees well with a $pK_a = 5.0 \pm 0.2$ estimated from the cation transport mediated by this ionophore at different pH values.[27] Furthermore, the p*Ka* of lauric acid agrees very well with the values reported for other fatty acids incorporated into phospholipid vesicles.[42] It appears to further justify the choice of the above solvent mixture as a model for the membrane interface.

Using fluorescent intravesicular pH probes such as sodium 1,3,6,8-pyrenetetrasulfonate has given us a direct proof of the coupling between Pr^{3+} transport and H^+ countertransport. Following entry of the paramagnetic cations, the intravesicular pH increases gradually. Joint addition of the ionophore *and of* lauric acid increases significantly this proton efflux, in full agreement with the mechanism earlier postulated.[2] These mea-

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surements will be reported in detail elsewhere.

Finally, one could question the physiological relevance of these experiments and observations. However, Pr^{3+} is an often-used Ca^{2+} substitute.[43] Also, and although free-acid acids are minor components of biological membranes, exogeneous additions alter several membrane-mediated cellular functions such as cell permeability[44] or activity of membrane-bound enzymes.[45-47] Furthermore, free fatty acids can be formed within the membrane by the action of phospholipases.

To sum up, the increase of Pr^{3+} translocation across phosphatidylcholine vesicles by lasalocid A, as induced by the single adjunction of lauric acid, finds its most likely explanation in participation of the fatty acid in the proton countertransport. Such a cation-proton antiport process could be used to model cation-proton exchanger systems in biological membranes.[47-50]

Registry No. X-537A, 25999-31-9; Pr, 7440-10-0; H^+, 12408-02-5; Na, 7440-23-5; lauric acid, 143-07-7.

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ENDOR of the Resting State of Nitrogenase Molybdenum-Iron Proteins from *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and *Clostridium pasteurianum*: ^{1}H, ^{57}Fe, ^{95}Mo, and ^{33}S Studies

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Abstract: Electron nuclear double resonance (ENDOR) studies of native and isotopically enriched MoFe proteins hold the promise of individually characterizing every atom of the catalytically active FeMo-co cluster of the nitrogenase MoFe protein. This report presents ^{1}H, ^{57}Fe, $^{95,97}Mo$, and ^{33}S ENDOR measurements in a comparison of the MoFe protein isolated from the three titled organisms, Avl, Kpl, and Cpl. We have examined in detail single-crystal-like ^{57}Fe resonances from at least five distinct iron sites in each of the three enzymes, revising somewhat our earlier assignments. The analysis incidentally gives the electron spin zero-field splitting parameters to high precision. ^{95}Mo ENDOR measurements for Cpl and Kpl give ^{95}Mo hyperfine and quadrupole coupling constants. They indicate that a single molybdenum is integrated into the MoFe spin system and that the molybdenum is most plausibly viewed as being in an even-electron state, which may be assigned provisionally as unsymmetrically coordinated Mo^{IV}. The observation of an exchangeable proton or protons from each protein source suggests a site on the cluster accessible to solvent and perhaps containing H_2O or OH^-. Cpl enriched in ^{33}S gives the first observed ENDOR signals from this nucleus. The resonances from ^{33}S are assignable to the inorganic sulfur because, it is argued, the FeMo-co cluster must be bound to the protein primarily, if not exclusively, by residues other than cysteinyl.

Nitrogenase comprises two extremely oxygen-sensitive metalloproteins, the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein).[2] The Fe protein accepts electrons from oxidative processes operating at or below -0.4 V and then specifically passes them on to the MoFe protein for use in the reduction of dinitrogen and other substrates.[3] The MoFe protein as isolated from *Azotobacter vinelandii* (Avl),[4] *Clostridium pasteurianum* (Cpl),[5,6] and *Klebsiella pneumoniae* (Kpl)[7] is an $\alpha_2\beta_2$ tetramer with molecular weights of approximately 240 000 for Avl and 220 000 for both Cpl and Kpl. Chemical analysis of the MoFe protein has shown it to contain 2 mol of molybdenum, approximately 30 mol of iron, and approximately 30 mol of acid-labile sulfur/mol of peptide component.[8]

Accurate chemical and spectroscopic assignment of these molybdenum, iron, and sulfur atoms into individual clusters and investigation of the structure of those clusters constitute much of the recent progress in the study of nitrogenase. In particular,

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(2) The following abbreviations are used: Avl, Kpl, and Cpl, the molybdenum-iron proteins, respectively, from *A. vinelandii*, *K. pneumoniae*, and *C. pasteurianum*; MoFe, molybdenum-iron protein; FeMo-co, molybdenum-iron cofactor; ENDOR, electron nuclear double resonance.
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