Israel National Council for Research and Development. We are very grateful to Prof. V. M. Schurig and D. Wistuba of the Institut für Organische Chemie, Tübingen, for analyses of cyclic ethers performed by complexation gas chromatography. Dr. Z. Ludmer of the Israel Institute for Biological Research is acknowledged for his gift of 100% hydrogen peroxide. We thank Dr. B. Maurer of Firmenich, Geneva, and Prof. D. Seebach of ETH, Zürich, for an authentic sample of compound **20**.

Catalytic Oxidation of Dithiols by a Semisynthetic Enzyme

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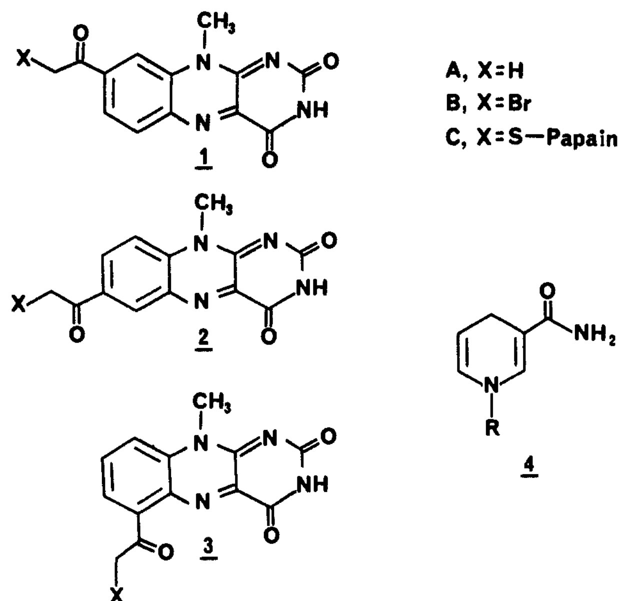
Abstract: The semisynthetic enzyme flavopapain (**1C**), obtained from the alkylation of Cys-25 of papain with 8α-(bromoacetyl)-10-methylisoalloxazine (**1B**), was found to be an effective catalyst for the oxidation of dithiols to disulfides. The k_2/K_s values for the oxidation of *d,l*-dihydrolipoamide and *d,l*-dihydrolipoic acid determined from anaerobic single-reaction stopped-flow kinetics were 4400 and 3400 M^{-1} s^{-1}, respectively. These values were, respectively, 126 and 200 times larger than the second-order rate constants for oxidation of *d,l*-dihydrolipoamide and *d,l*-dihydrolipoic acid by the model flavin 8-acetyl-10-methylisoalloxazine (**1A**). Under aerobic turnover conditions using the synthetic dye MTT as an electron acceptor, the k_{cat} and K_m values for the oxidation of *d,l*-dihydrolipoamide by **1C** were in approximate agreement with the k_2 and K_s values, indicating that the rate-limiting step of the catalytic cycle is substrate oxidation rather than oxidation of dihydroflavopapain. When compared with flavopapains **2C** and **3C** [obtained as above but by modification with 7α- and 6α-(bromoacetyl)-10-methylisoalloxazine (**2B** and **3B**, respectively)], flavopapain **1C** is the most efficient catalyst. The circular dichroic spectra of flavopapains **1C**, **2C**, and **3C** were recorded, and the dissociation constants of the sulfite addition complexes of **1C** and **2C** were determined. From these kinetic and physical studies, the differences in catalytic activity of **1C**, **2C**, and **3C** were judged to be due to changes in the flavin orientation within the active site and the ability to fit the substrate into a productive reaction conformation.

The chemical modification of existing protein molecules to produce new catalysts represents an important approach to enzyme engineering.[1] In our work, we have covalently linked isoalloxazine derivatives to proteolytic enzymes such as papain to create new redox enzymes.[2] Specifically, the Cys-25 thiol of papain was treated with α-(bromoacetyl)isoalloxazines **1B**, **2B**, and **3B** to produce "flavopapain" semisynthetic enzymes **1C**, **2C**, and **3C**, respectively[3] (Chart I). These flavopapains have been shown to be catalysts for the oxidation of *N*-alkyl-1,4-dihydronicotinamides **4**. When coupled with artificial electron acceptors, flavopapain **1C** has been shown to be a particularly efficient oxidation catalyst.[4]

In view of papain's broad specificity as a hydrolytic enzyme, it appeared likely that the flavopapain enzymes might bind other classes of substrates and catalyze different kinds of redox reactions. Among the many oxidation reactions catalyzed by flavins is the oxidation of thiols to disulfides. Indeed, flavopapain **2C** was shown to mediate the oxidation of dithiols, albeit with only modest rate acceleration relative to the model flavin compound **2A**.[5] We now report the first demonstration of efficient turnover catalysis of thiol oxidation by a synthetic catalyst, flavopapain **1C**. We also report on the results of several studies designed to aid in understanding the molecular basis of the different catalytic efficiencies of flavopapains **1C**, **2C**, and **3C**.

Chart I



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Experimental Section

Instrumentation. Scanning UV–visible spectrophotometry was carried out on either a Perkin-Elmer λ-5 or a Varian Cary 219 spectrophotometer equipped with thermostated cell compartments. Stopped-flow measurements were made on a Durrum-Gibson stopped-flow spectrophotometer with a thermostated syringe compartment. All solutions were preequilibrated in the thermostated compartments for 5 min prior to recording data. Circular dichroism spectra were recorded on an AVIV 60DS CD spectrometer. High-pressure liquid chromatography analyses were performed on a Waters Associates liquid chromatograph equipped with a Altex reversed-phase G-18 analytical column.

Materials. *d,l*-Lipoamide and *d,l*-dihydrolipoic acid were purchased from Sigma. *d,l*-Dihydrolipoamide was prepared according to literature

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procedures[6] and stored desiccated at 4 °C. Papain (Worthington Biochemicals) was purified by affinity chromatography on a column of Gly-Gly-Tyr-Arg-agarose.[7] Freshly purified papain was treated with a 5-fold excess of (bromoacetyl)flavins **1B**, **2B**, or **3B** (as concentrated solutions in dimethyl sulfoxide) in the dark to produce flavopapains **1C**, **2C**, or **3C**, respectively, following the procedure described previously.[1b,3] The chemical modification was monitored by measuring the loss of papain hydrolytic activity in the rate of hydrolysis of *N*-benzoyl-D,L-arginine-*p*-nitroanilide.[8] After dialysis against 50 mM NaCl (4 °C, protected from light, three changes of solution over 48 h) to remove unreacted flavin, the concentrations of flavopapains **1C**, **2C**, and **3C** were determined by using the molar extinction coefficients of flavins **1A**, **2A**, and **3A** (**1A**, ϵ_{443} = 11 000, ϵ_{280} = 18 800; **2A**, ϵ_{427} = 10 900, ϵ_{280} = 32 300; **3A**, ϵ_{433} = 10 500, ϵ_{280} = 11 300).[1b,3] The molar extinction coefficients of the flavopapains at wavelengths greater than 300 nm were assumed to be equal to those of the model flavins. The amount of protein was calculated from the absorbance at 280 nm, using values of $\epsilon^{1\%}_{280}$ = 25.0 and a molecular weight of 23 400,[9,10] after substracting the contribution to the absorbance from the flavin. In each case, the protein/flavin ratio in flavopapains **1C**, **2C**, and **3C** was 1.0 ± 0.1. The flavopapains could be stored at 4 °C for 1 month with only a small decrease in activity.

Kinetics. All kinetic measurements were carried out at 25.0 ± 0.3 °C. The anaerobic reactions between **1A** and *d,l*-dihydrolipoamide or *d,l*-dihydrolipoic acid were monitored by measuring the loss of flavin absorbance at 445 nm under pseudo-first-order conditions with dithiol in excess. In a typical experiment, 3 mL of a buffered solution (0.02 M Tris-HCl, 0.08 M KCl, 0.1 mM EDTA, pH 7.5) containing **1A** (9 μM, prepared by adding a concentrated solution of **1A** in dimethyl sulfoxide to buffer) was added to the main compartment of a Thunberg cuvette. The dithiol, dissolved in dimethyl sulfoxide, was added to the side arm of the cuvette. After deoxygenation of the cuvette by cycling the gas space six times between vacuum and argon, the dithiol solution was added from the cuvette side arm to initiate the reaction (the concentration of dithiol ranged from 0.1 to 1.0 mM). First-order decay of the flavin absorbance was observed for 3 half-lives. Upon completion of the reactions, admittance of air regenerated the oxidized flavin species quantitatively. A plot of the pseudo-first-order rate constant against dithiol concentration exhibited linearity, establishing that the reaction of **1A** and dithiol is first order in both reactants.

The rates of the rapid anaerobic reactions between **1C** and *d,l*-dihydrolipoamide and *d,l*-dihydrolipoic acid were measured by loading degassed (held in vacuo for 20 min prior to loading) solutions of **1C** and dithiol into the drive syringes of the stopped-flow spectrometer. After the solutions were mixed, the loss of absorbance at 445 nm due to reduction of the flavopapain (4 μM) was monitored under pseudo-first-order conditions with dithiol in excess (0.2–6 mM). Unlike the kinetics of dithiol oxidation shown by **1A**, the pseudo-first-order rate constants determined with **1C** plotted against dithiol concentration did not exhibit linearity but yielded a curve typical of saturation kinetics. The double-reciprocal plot exhibited linearity from which the k_2 and K_s values could be determined.[11]

The turnover kinetics for the reaction between *d,l*-dihydrolipoamide and **1C** were measured by using 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT, Aldrich) as the artificial electron acceptor.[12] In a typical experiment, 2 mL of a buffered solution (0.02 M Tris-HCl, 0.08 M KCl, 0.1 mM EDTA, pH 7.5) containing dithiol (0.1–4.3 mM) and MTT (17 μM) was placed in a 3-mL cuvette. An aliquot of a solution containing **1C** (final concentration 0.084 μM) was added, and the increase in absorbance at 560 nm was followed (MTT reduction product, ϵ_{560} = 14 000). At the concentration of flavopapain used, an increase in concentration of MTT did not alter the derived kinetic parameters. Thus, the reaction rate was established to be zero order with respect to MTT concentration. The rate of the background reaction between MTT and dihydrolipoamide (approximately 10–20% of the flavopapain-catalyzed rate) was determined under conditions identical with the above conditions without enzyme. This background reaction rate was subtracted from all rate measurements.

Product Isolation Experiments. Under conditions similar to those used in the kinetic experiments, 0.2 μmol of **1C** in 14 mL of deoxygenated buffer (0.02 M Tris-HCl, 0.08 M KCl, 0.1 mM EDTA, pH 7.5) was allowed to react with 0.2 μmol of dihydrolipoamide (added as a solution in deoxygenated methanol). After 60 s, 20 mL of deoxygenated dichloromethane was added. After the mixture was stirred for 1 min, the organic layer was separated and evaporated under reduced pressure. The residue was taken up in methanol and analyzed by high-pressure liquid chromatography (60% water, 40% methanol).

Table I. Catalytic Rate Parameters for the Oxidation of Dithiols by Flavopapains **1C** and **2C** and Flavins **1A** and **2A**[a]

oxidant	substrate	k_2, s^{-1}	K_s, mM	k_2/K_s, M^{-1} s^{-1}	k_{model}, M^{-1} s^{-1}
1C	dihydrolipoamide	10 (4[b])	2.2 (3.7[b])	4400	35
2C[c]	dihydrolipoamide	~1	~50	21	1.2
1C	dihydrolipoic acid	1.4	0.42	3400	17
2C[c]	dihydrolipoic acid			6.7	0.8

[a] All values refer to anaerobic conditions where a single reaction was observed [25 °C, 0.02 M Tris-HCl, 0.08 M KCl, 0.1 mM EDTA, 3–6% dimethyl sulfoxide, pH 7.5] except where noted. The values of k_{model} refer to the model flavin reactions of either **1A** or **2A**. [b] Values in parentheses refer to aerobic turnover conditions with MTT as the electron acceptor. K_s corresponds to the K_m value for dihydrolipoamide, and k_2 corresponds to k_{cat}. [c] Values taken from ref 5.

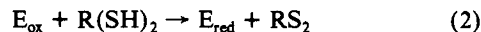
Results and Discussion

Kinetics. The oxidation of *d,l*-dihydrolipoic acid and *d,l*-dihydrolipoamide by flavin **1A** was found to be first order in both species. This is in agreement with the currently accepted mechanism of flavin oxidation of thiols.[13] It is interesting that there is a 20–30-fold increase in reactivity of flavin **1A** over **2A**. This type of influence of substituents upon flavin reactivity has been observed for other flavins[14] and is possibly related to the direct quinoid conjugation of the enol form of the acetyl group with the flavin ring system[15]—an interaction possible with **1A** but not with **2A**. The oxidation of the same dithiols by **1C** was observed to exhibit saturation kinetics. As outlined in eq 1, the basic kinetic

$$E_{ox} + R(SH)_2 \underset{K_s}{\rightleftharpoons} E_{ox}{\cdot}R(SH)_2 \xrightarrow{k_2} E_{red} + RS_2 \quad (1)$$

scheme shows dithiol reversibly binding to flavopapain to form a Michaelis complex that breaks down in a product-determining step (with rate constant k_2), where E_{ox} and E_{red} are the oxidized and reduced forms of flavopapain and $R(SH)_2$ and RS_2 are the reduced and oxidized forms of dithiol substrates. The product-forming step represented by k_2 undoubtedly involves a more complicated set of steps, including formation of a flavin **4a** adduct followed by disulfide bond formation and product release.[13] The rate parameters for anaerobic dithiol oxidation by flavins **1A** and **2A** and flavopapains **1C** and **2C** are shown in Table I.

The above kinetic analysis has focused only on the first half of a potentially catalytic cycle: the oxidation of substrate with the concomitant reduction of flavopapain. Any catalytic cycle must include reoxidation of reduced flavopapain in a second step to regenerate oxidized flavin as shown in eq 2 and 3, where A_{ox}



$$E_{ox} + R(SH)_2 \rightarrow E_{red} + RS_2 \quad (2)$$

$$E_{red} + A_{ox} \rightarrow E_{ox} + A_{red} \quad (3)$$

is an electron acceptor and A_{red} is its reduced form. While it is common for flavoenzymes to use dioxygen as the electron acceptor, it is known that the reaction between dioxygen and reduced flavopapain is slow and partially rate determining in the catalytic oxidation of dihydronicotinamide substrates.[4] Synthetic electron acceptors such as the dye MTT were shown to be suitable electron acceptors, resulting in efficient catalytic oxidation of dihydronicotinamides.[4] In the oxidation of dithiols, MTT was also found

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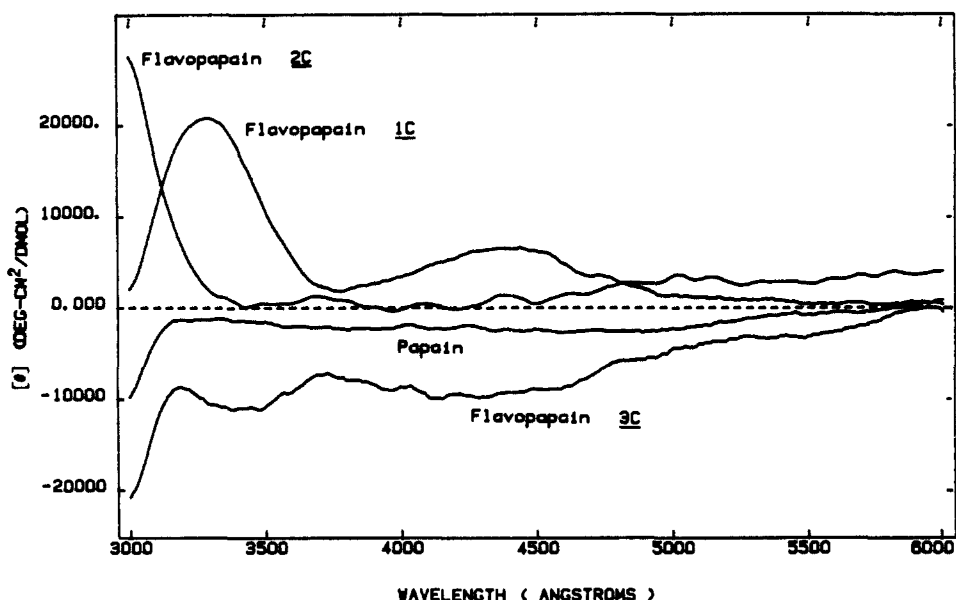

Figure 1. Circular dichroic spectra of papain and flavopapains **1C**, **2C**, and **3C** (25 °C; pH 6.5; 50 mM NaCl): **1C**, 35 μM; **2C**, 16 μM; **3C**, 19 μM; papain, 36 μM.

to be a suitable artificial electron acceptor for use in turnover kinetic studies. The k_{cat} and K_m parameters determined from turnover kinetics, shown in Table I, are in reasonable agreement with the values of k_2 and K_s determined from the single reaction kinetics. This indicates that the rate-limiting step of the catalytic cycle is probably oxidation of substrate (eq 2) rather than oxidation of the reduced form of flavopapain (eq 3).

Semisynthetic Enzyme Efficiency and Substrate Selectivity. Flavopapains **1C** and **2C** were found to catalyze the efficient oxidation of many dithiols such as dithiothreitol, 2,3-butanedithiol, 2,3-dimercapto-1-propanol, and 2,3-dimercaptopropanesulfonic acid. Dihydrolipoamide and dihydrolipoic acid were chosen as substrates on which to focus the kinetic determinations becuase of their biological interest as cofactors for enzymes of the pyruvate dehydrogenase complex.[16] The fact that a stopped-flow spectrometer was necessary to measure the anaerobic rate parameters for **1C** while conventional spectrophotometric procedures sufficed for the kinetic analysis of the catalysis shown by **2C** is a rough indication of the substantially greater efficiency of **1C** as a catalyst. A direct comparison of the rate accelerations for the two semisynthetic enzymes **1C** and **2C** relative to model reactions clearly shows the increase in efficiency for **1C**. For the oxidation of dihydrolipoic acid by flavopapain **1C**, the k_{cat}/K_m value of 3400 $M^{-1} s^{-1}$ is 200 times larger than the k_2 value of the model reaction (flavin **1A**) of 17 $M^{-1} s^{-1}$. This enzymatic rate acceleration is much larger than the 8-fold acceleration shown by **2C**.

From the experiments performed, there appeared to be no evidence for the preferential enzymatic oxidation of one of the enantiomers of the two substrates studied. Thus, efficient catalysis of thiol oxidation by **1C** has been demonstrated even though the binding site is not optimally designed for dihydrolipoyl dithiol substrates. Yeast lipoamide dehydrogenase, part of the pyruvate dehydrogenase complex,[16] mediates the oxidation of L-dihydrolipoamide.[17] A comparison of the kinetic parameters for the oxidation of *d,l*-dihydrolipoamide by **1C**, $k_{cat} = 4\ s^{-1}$ and $K_m =$ 3.7 mM, with those for oxidation of L-dihydrolipoamide by lipoamide dehydrogenase,[18] $k_{cat} = 430\ s^{-1}$ and $K_m = 0.7$ mM, shows that the catalytic efficiency of **1C**, although impressive, is still appreciably lower than that of the naturally occurring enzyme.

The trend in efficiency of dithiol oxidation by flavopapains is **1C** > **2C** > **3C**.[19] It is instructive to make a rough comparison with the flavopapain-catalyzed oxidation of *N*-alkyldihydronicotinamides. For example, k_{cat} and K_m for the oxidation of *N*-benzyl-1,4-dihydronicotinamide by flavopapains **1C** and **3C** with MTT as the electron acceptor are 29 s^{-1}, 0.47 mM and 0.03 s^{-1}, 0.40 mM, respectively (pH 7.5, 0.1 M Tris, 0.1 mM EDTA).[4,20] In the presence of MTT, the k_{cat} and K_m parameters for the oxidation of *N*-propyl-1,4-dihydronicotinamide by flavopapains **1C** and **2C** are 31 s^{-1}, 0.21 mM and 58 s^{-1}, 0.70 mM, respectively (pH 7.5, 0.1 M Tris, 0.1 mM EDTA).[4,20] The k_{cat}/K_m ratios in the catalytic oxidation of dihydronicotinamides with MTT as the electron acceptor show that **1C** is slightly more effective than **2C**, which in turn is much more effective than **3C**. Thus, for two kinds of substrates, dithiols and dihydronicotinamides, the same trend in catalytic efficiency of flavopapain is observed. To investigate further this structural dependence of activity, the circular dichroism spectra of **1C**, **2C**, and **3C** were recorded and are shown in Figure 1 along with that of papain. As is typical for flavoenzymes, the chiral environment of the enzyme's active site perturbs the flavin chromophore so as to cause differential absorbance of left and right circularly polarized light.[21] Control experiments in which **1A**, **2A**, or **3A** was added to papain in approximately equimolar amounts generated CD spectra not significantly different from the spectrum of papain. It is very interesting that the spectrum of each of the flavopapains is different: **1C** shows two positive bands at 440 and 330 nm, **2C** shows one positive band at 300 nm, and **3C** shows no positive bands. Apparently, the transition dipoles of the three flavin chromophores must be oriented in different directions. Since each flavopapain exhibits a flavin-dependent circular dichroic spectrum, it is likely that the flavin group within the active site of papain in **1C**, **2C**, and **3C** is conformationally restricted. Given the trend in catalytic efficiency, **1C** must have the flavin group held in the most productive orientation for reaction with substrate. Addition of the tetrapeptide Gly-Gly-Tyr-Arg (7 mM), a known inhibitor of papain,[7] did not induce a shift in the circular dichroic spectrum of **1C** (0.035 mM, pH 6.5).

The formation of a reversible N-5 sulfite adduct has been used as one criterion in the classification of naturally occurring flavoproteins.[22] Flavoenzymes possessing oxidase activity have been observed to have high reactivity with sulfite, in fact, much higher

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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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Contribution from the Institut de Chimie Organique et de Biochimie, Université de Liège au Sart-Tilman, 4000 Liège, Belgium. *Received October 16, 1985*

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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