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Spontaneous Phospholipid Transfer: Development of a Quantitative Model†

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ABSTRACT: The effects of lipid structure on the kinetics of spontaneous transfer of a series of phosphatidylcholines have been determined. Donors, which were model-reassembled high-density lipoproteins composed of apo A-I, 1-palmitoyl-2-oleoylphosphatidylcholine, and a trace of a radiolabeled lipid, were mixed with acceptors, which were human low-density lipoproteins. Within a series of phosphatidylcholines, the addition of double bonds and methylene units, respectively, increased and decreased the rate of transfer in a predictable way. An equation that predicts the rates of transfer of a large number of diacylglycerides and phosphoglycerides from any lipoprotein has been empirically derived from these data. The transfers of phosphatidylcholines that contain superpolyunsaturated fatty acids (four or more double bonds) do not obey the derived equation, probably due to limitations on the number of conformational degrees of freedom in these lipids. The range of measured transfer halftimes extends from less than 2 h to more than 12 days. Thus, the spontaneous transfer halftimes of some (but not all) lipids are short compared to the lifetime of lipoproteins in plasma. These results suggest that some lipids transfer among lipoproteins and cells via a spontaneous mechanism while others require specific transfer factors or hydrolysis to achieve this within a physiologically significant time frame.

PCs[1] transfer between lipid surfaces by both spontaneous and protein-mediated processes (Roseman & Thompson, 1980; Massey et al., 1981, 1982, 1984; Nichols & Pagano, 1981; Wirtz et al., 1972; McLean & Phillips, 1981). The former process is highly sensitive to the structure of the transferring species and its microenvironment. Additional double bonds or methylene units in the acyl chains increase or decrease,

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[1] Abbreviations: Phosphatidylcholine, PC; 1-lauroyl-2-oleoyl-PC, LOPC; 1-myristoyl-2-oleoyl-PC, MOPC; 1-palmitoyl-2-oleoyl-PC, POPC; 1-palmitoyl-2-stearoyl-PC, PSPC; 1-palmitoyl-2-linoleoyl-PC, PLnPC; 1-palmitoyl-2-linolenoyl-PC, PLinPC, 1-palmitoyl-2-arachidonyl-PC, PAPC; 1-palmitoyl-2-eicosapentaenoyl-PC, PEPC, 1-palmitoyl-1-docosahexaenoyl-PC, PDhPC; 1,2-dioleoyl-PC, DOPC; low-density lipoproteins, LDL; high-density lipoproteins, HDL; model-reassembled HDL, R-HDL.

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respectively, the rate of spontaneous transfer by a predictable increment (Massey et al., 1981, 1984). In contrast, the spontaneous transfer rate increases with decreasing Stokes radius of the donor particle. Many of these correlations were based upon studies in which the transfer of fluorescent lipids was measured in a variety of lipid environments in which the number of double bonds did not exceed two (Massey et al., 1981). Herein, we derive a set of rules that applies to physiologically abundant PCs. A single equation that can be used to predict the relative rates of spontaneous transfer of a large number of similarly structured molecules is provided.

Materials and Methods

LDL were isolated by sequential ultracentrifugation at d = 1.006 and d = 1.063 g/mL. The isolated LDL were further purified by chromatography on heparin-Sepharose to remove any lipoproteins that do not bind to heparin. The acceptor LDL were then removed with high salt. Apo A-I was isolated from normal human subjects as previously described (Pownall et al., 1979). [^{3}H]DOPC and [^{3}H]PAPC were obtained from New England Nuclear. Other radiolabeled phospholipids were formed by methylation of the corresponding phosphatidylethanolamine with [^{3}H]methyl iodide (Patel et al., 1979). R-HDL, which were prepared by the cholate removal method of Matz and Jonas (1982), were used as donors. The particles contained lipid and protein in a molar ratio of 100:1, the amount of radiolabeled lipid never exceeded 10%. During synthesis and in the tests that used PAPC, PEPC, or PDhPC, butylated hydroxytoluene (10 μg/mL) was included to retard lipid peroxidation. Kinetics studies of these lipids were conducted within 24 h of synthesis, and oxygen was excluded by N_2 purgation.

For the lipid transfer assay, a system composed of R-HDL (donors) and human LDL (acceptors) was selected for two reasons. First, the donors and acceptors can be easily separated by a simple assay. Second, a wide variety of labeled lipids can be incorporated into R-HDL composed mostly of one lipid with little alteration of the particle structure. We selected a series of diacyl PCs in which the fatty acyl groups at the *sn*-1 and *sn*-2 positions, respectively, are similar to natural PCs, i.e., a saturated fatty acid at *sn*-1 and an unsaturated fatty acid at *sn*-2.

The transfer assay was a modification of that of Ellsworth et al. (1982) except that in our assay the donors were composed of R-HDL and the acceptors were human LDL. Experiments were initiated by mixing R-HDL (2 μmol of PC in 0.3 mL) and LDL (25 μmol of phospholipid in 2.7 mL) at 37 °C. At appropriate time intervals, a sample was removed and added to a 3–4-fold excess of ice-cold low-salt buffer (50 mM NaCl, 10 mM Tris, pH 7.4), and the HDL and LDL were separated on a 1.5-mL column of heparin-Sepharose in a Quick-Sep column (Isolabs). The HDL and LDL, respectively, were eluted with 2 mL of ice-cold low-salt buffer and 500 mM NaCl and 10 mM Tris, pH 7.4. Both acceptor and donors were counted and the rate constants for transfer determined by a computer program using

$$C(t) = A + B \exp(-kt)$$

in which $C(t)$ is the transferred number of counts at time t, A is the value of $C(t)$ at infinity, B is a preexponential, k is the first-order rate constant, and t is the time in minutes. Similar rate constants were obtained when the appearance of label in the acceptor or its disappearance in the donor was monitored. At the end of the experiments, high-performance liquid chromatography revealed that >96% of the radioactivity was still associated with the PC peak.

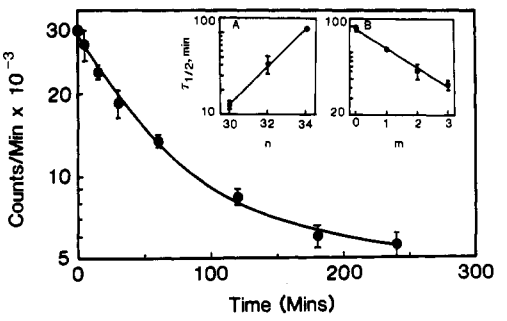

FIGURE 1: Representative kinetic data for the transfer of PLinPC from R-HDL (0.67 mM PC) to LDL (8.3 mM phospholipid) at 37 °C. (Insert A) Correlation of the rate constant for spontaneous transfer with the total number of carbons in the acyl chains at the *sn*-1 and *sn*-2 position of a series of PCs in which the *sn*-2 position contains an oleoyl group and the saturated chain at *sn*-1 is varied from 12 to 16 carbons. R-HDL and LDL were the donors and acceptors, respectively. The transfer temperature was 37 °C. Error bars are the standard deviation. (Insert B) Correlation of the rate constant for spontaneous transfer with the number of double bonds, m. The 18-carbon acyl chain at the *sn*-2 position of a series of 1-palmitoyl-2-acyl-PCs contains m cis double bonds. R-HDL and LDL were the respective donor and acceptor particles. The experimental temperature was 37 °C. Error bars are the standard deviation.

Table I: Halftimes (min) for Phosphatidylcholine Transfer

	sn-1/*sn*-2[a]	calcd[b]	measured	$\delta\Delta G^{o\ddagger}$ (cal/mol)[c]
LOPC	12:0/18:1	12	11 ± 1	50
MOPC	14:0/18:1	35	38 ± 3	−49
POPC	16:0/18:1	102	95 ± 2	43
PSPC	16:0/18:0	157	145 ± 7	48
PLnPC	16:0/18:2	66	68 ± 7	−18
PLinPC	16:0/18:3	42	38 ± 6	60
PAPC	16:0/20/4	81	175 ± 20	−460
DOPC	18:1/18:1	191	211 ± 20	−60
PEPC	16:0/20:5 (n − 3)	52	155 ± 10	−650
PDhPC	16:0/22:6 (n − 3)	100	382 ± 60	−800

[a] n:m represents the identity of acyl chains at the *sn*-1 and *sn*-2 position; n is the number of carbons in the acyl chain and m is the number of double bonds. [b] From eq 5a. [c] $\delta\Delta G^{o\ddagger}$ is the difference between the calculated and measured free energies of activation for spontaneous transfer. This is calculated from $\delta\Delta G = RT[\ln \tau_{1/2(calc)} - \ln \tau_{1/2(obsd)}]$.

Results

A typical plot of the transfer of PLinPC from R-HDL to LDL is shown in Figure 1. Similar experiments with other PCs and the spontaneous transfer halftimes of a series of PCs with varying acyl chain lengths were determined at 37 °C (Table I). A plot of the log of the halftime or rate constant versus the number of carbons in the acyl chain is linear (Figure 1, Insert A) and is consistent with each methylene unit contributing a constant increment to the free energy of activation. Similarly, the addition of each cis double bond to a series of PCs in which the *sn*-1 position contains a palmitoyl moiety and the *sn*-2 position contains an 18-carbon acyl chain in which the unsaturation is varied reduces the transfer time by a constant fraction (Figure 1, insert B). This corresponds to each double bond contributing a constant decrement to the free energy of activation.

Transfer rate dependence on the number of methylene units and double bonds in the lipid correlates with the effect these structures have on the equilibrium constant for the partitioning of hydrocarbon-containing molecules between water and a hydrocarbon phase (Nichols, 1985; Tanford, 1972). Each additional double bond increases the aqueous phase solubility, while each additional methylene unit decreases the solubility of a homologous series by a constant fraction. Therefore, the transition state for lipid transfer is similar, if not identical, to

that of bulk phase water (Pownall et al., 1983). From absolute rate theory (Moore, 1964), the free energy of an activation ($\Delta G_i^{o\ddagger}$) for the ith homologue in a series is given by

$$\Delta G_i^{o\ddagger} = -RT \ln (k_i h/kT) \quad (1)$$

where R is the gas constant, k_i is the rate constant, k is the Boltzmann constant, T is the absolute temperature, and h is Planck's constant. The difference in the free energy of transfer of a pair of homologues A and B is given by

$$\Delta\Delta G_{AB}^{o\ddagger} = \Delta G_A^{o\ddagger} - \Delta G_B^{o\ddagger} \quad (2)$$

Substituting eq 1 into eq 2 gives

$$\Delta\Delta G = RT \ln k_A/k_B = 2.3RT \log k_A/k_B \quad (3a)$$

$$= 2.3RT \log \tau_{B1/2}/\tau_{A1/2} \quad (3b)$$

where $\tau_{1/2}$ is the halftime. If each methylene unit contributes a constant increment to the free energy of activation, a semilogarithmic plot $\tau_{1/2}$ versus the number of methylene units in the acyl chain should be linear with a slope corresponding to $\Delta\Delta G^{o\ddagger}/2.3RT$. The value of $\Delta\Delta G^{o\ddagger}$ calculated from Figure 1 (insert A) is 0.32 kcal/mol methylene unit. The equation for this line is

$$\log \tau_{1/2} = 0.234n - 5.95 \quad (4a)$$

where $\tau_{1/2}$ is given in minutes; in terms of a rate constant, we have

$$\log k_i = 5.7 - 0.234n \quad (4b)$$

where n is the number of carbon atoms in a straight acyl chain. A similar calculation for m double bonds, which was based on the data of Figure 1 (insert B), gave $\Delta\Delta G^{o\ddagger} = -0.30$ kcal/mol double bond. This effect can be combined with that of acyl chain length to give an equation that relates transfer halftime to PC structure, i.e.

$$\log \tau_{1/2} = 0.234n - 0.189m - 5.76 \quad (5a)$$

or

$$\log k_i = 5.5 + 0.189m - 0.234n \quad (5b)$$

As one test of the validity of eq 5b, the calculated and measured transfer rates of DOPC, which was not used to determine the coefficients on n and m, were compared (Table I). These data show that there is a very close correspondence between these values, suggesting that eqs. 5a and 5b can be used to predict the rates of spontaneous transfer of some PCs with an error that is on the order of 10%. Although equations 5a and b were based upon PC transfer where R-HDL was the donor, the rate of spontaneous transfer of other diacylglycerides from other donors may be predicted by using linear free energy relationships. Assuming that each part of the transferring molecule and its microenvironment contribute independently to the partitioning of the molecule between the membrane and the activated state, one can derive a master equation that predicts the halftime of spontaneous transfer of all of the common diacylglycerides and phosphoglycerides.

The general equation, which is an expansion of equations 5a and 5b, is

$$\log \tau_{1/2} = 0.234n - 0.189m + C_1 + C_2 \quad (6)$$

where C_1 and C_2 represent the contributions of the polar headgroup and donor surface structure.

Comparing eq 6 to eq 5a, which predicts the rate of transfer of a PC from R-HDL to LDL, we determine that $C_1 + C_2 = -5.76$. If transfer from R-HDL is taken as a reference state ($C_2 = 0$), the effects of replacement of the phosphorylcholine ($C_1 = -5.76$) with other functional groups can be calculated from the data of Massey et al. (1982), who observed that substitution of phosphorylcholine with hydroxyl, phosphorylethanolamine, phosphorylserine, phosphorylglycerol, and phosphate decreased the transfer rates by factors of 19, 3.7, 1.7, 2.6, and 1.3. In terms of their logarithms, the respective values of C_1 are reduced by 1.28, 0.56, 0.23, 0.41, and 0.11, giving the values of C_1 listed in Table II.

Table II: Polar Group Constants for Individual Lipid Classes

	C_1[a]	$\tau_{1/2}/\tau_{1/2}$ (PC)[a]
diacylglycerol	−4.46	20
phosphatidic acid	−5.65	1.3
phosphatidylcholine	−5.76	1.0
phosphatidylethanolamine	−5.19	3.7
phosphatidylglycerol	−5.34	2.6
phosphatidylserine	−5.54	1.7

[a] Calculated from the data of Massey et al. (1982), in which the incremental increase or decrease in rate occurs when the polar headgroup of PC is replaced by another functional group.

Table III: Macromolecular Constants for the Donor Particle

	C_2	10^{C_2}
R-HDL (POPC/apo A-I, 100:1)	0.0	1
POPC single-bilayer vesicles	0.7	5
DMPC single-bilayer vesicles	0.8	6
HDL	0.44	3
LDL	0.80	6
VLDL	1.19	16

Finally, it has been observed that PCs desorb from lipid surfaces at a rate that is a function of the "quality" of the interface. For example, the ratios, R, of the halftimes of transfer of 1-myristoyl-2-pyrenylnonanoyl-PC from R-HDL, HDL, LDL, and VLDL are 1:2.8:6.3:16 (Massey et al., 1984). Similar ratios have been reported for other lipids (Lund-Katz et al., 1982), so the validity of eq 6 probably extends beyond the series of PCs tested herein. With $C_2 = \log R$, we obtain the values of C_2 that are given in Table III.

The Anomalous Behavior of Superpolyunsaturated PCs. In Table I, the measured and calculated values are compared; with the exceptions of PAPC, PEPC, and PDhPC, which were not part of the series used to generate eq 5, a good correspondence between experimental and calculated values is observed; the average deviation between the observed halftimes and the halftimes calculated from eq 6 was 8%. These can be expressed in terms of an average deviation in energy from eq 3, which may be rearranged to give

$$\delta\Delta G^{o\ddagger} = \Delta G_{obs}^{o\ddagger} - \Delta G_{calc}^{o\ddagger} = RT \ln k_{obs}/k_{calc} = -RT \ln t_{1/2(obs)}/t_{1/2(calc)} \quad (7)$$

Equation 7 was used to calculate the difference in the observed and calculated free energy for spontaneous transfer. These data are summarized in Table I and show that there is a very small difference in the calculated and observed free energies of activation for lipid transfer for all of the PCs carrying up to 3 double bonds and 18 carbons in the largest chain. This is certainly within experimental error of the technique used to measure kinetics. By contrast, the transfer rates and associated differences in the observed and calculated free energies of activation for the transfer of PAPC, PEPC, and PDhPC are far different. For PAPC, PEPC, and PDhPC, the measured times are 216, 298, and 382%, respectively, of those calculated from eq 6. Inspection of the corresponding values of $\delta\Delta G^{o\ddagger}$ shows that the energy differences, though small, are far from insigificant.

Discussion

Structural effects exerted by the introduction of double bonds into PCs include intermolecular motion (Stubbs et al.,

1981), surface viscosity (Evans et al., 1980, 1981), and molecular surface area (Evans et al., 1987). The magnitude of each effect is greatest with the first added double bond; therefore, it is unlikely the anomalous transfer rates observed when four or more double bonds were in the *sn*-2 acyl chain were due to any of these effects.

The data of Figure 1 (insert A) show that each methylene unit contributes 320 calories to the free energy of activation so that, in the case of PDhPC, the difference in the observed and calculated transfer rates corresponds to the effects of 800/320 or 2.5 methylene units. Thus, it appears that the superpolyunsaturated fatty acids contribute more hydrophobic free energy to the free energy of activation, while the lower homologues contribute less. Comparison of our data with the large body of kinetic and thermodynamic data supports this viewpoint. Previous data on the transfer of single-chained amphiphiles demonstrated that each methylene unit contributed 700–800 cal/CH_2 to both the free energy of activation (Pownall et al., 1983; Massey et al., 1984) and the free energy of transfer (Tanford, 1972). This is approximately double the value observed in this study and in the reports on pyrene-labeled phospholipids (Massey et al., 1982). Similarly, Smith and Tanford (1972) reported that the incremental free energy of transfer from water to a lipid environment of a methylene unit in a two-chained amphiphile was less than that of a single-chained amphiphile with the same number of methylene units. This effect was assigned to the formation of hydrophobic bonds, which keep the acyl chains at the *sn*-1 and *sn*-2 positions of PC associated with each other even when the lipid is an aqueous monomer in water. As a consequence, the exposed hydrophobic area, which is a more reliable predictor of behavior than chain length, is smaller than expected. From this, it is inferred that the additional double bonds or chain length of PCs that contain a superpolyunsaturated fatty acid lead to the exposure of hydrophobic surfaces on one or both acyl chains so that their contribution to the free energy is more consistent with the number of methylene units in single-chained amphiphiles. We propose two possibilities. The first, which can be excluded on the basis of molecular models, is that the acyl chain at the *sn*-2 position has more carbons than the one located at *sn*-1 so it can protrude beyond the terminal methyl group of the chain at *sn*-1. This protrusion exposes the last few carbons, which are no longer shielded by contact with the *sn*-1 chain. However, in models of phospholipids in bilayers, a kink in the *sn*-2 chain aligns the C_1 carbon of the *sn*-2 chain with the C_4 of the *sn*-1 chain (Hauser et al., 1981). Furthermore, superpolyunsaturated fatty acids are actually shorter than saturated acyl chains with the same carbon number by virtue of double bond kinks. Therefore, the deviations of observed from calculated transfer rates are probably not due to differences in the extension of the *sn*-1 and *sn*-2 chains.

A second explanation, which we favor, is that the superpolyunsaturated fatty acids in PCs are kinked so that a greater fraction of the hydrophobic surface is exposed to the solvent when they are monomers in water. Applegate and Glomset (1986) have performed computer-based modeling studies of the structure of glycerols containing a stearoyl group at the *sn*-1 position and an arachidonoyl or docosahexaenoyl moiety at *sn*-2. Models of both lipids exhibit kinks near the end of the *sn*-2 acyl chain, an effect that is particularly prominent in the protrusion of the terminal pentyl moiety of arachidonic acid. The exposure of the terminal ethyl group of docosahexaenoic acid is less obvious and probably less effective in increasing the free energy of transfer of the molecule to water in the activated state. However, other irregularities and protrusions produced by the six cis double bonds may be large enough to effect the overall increase in exposed hydrophobic area.

Two conclusions may be drawn from these results. In terms of the mechanism of spontaneous lipid transfer, which occurs via the aqueous phase that separates donor and acceptors, our results provide an additional refinement that (1) quantifies structure with transfer rates and (2) reconciles the inconsistency in the incremental hydrophobicities of single- and doubled-chained amphiphiles. With respect to the physiological significance of spontaneous lipid transfer, an additional conclusion may be drawn; i.e., the importance of spontaneous transfer is dependent upon its rate relative to those for other processes. If the rate of spontaneous transfer from a given membrane or lipoprotein is on the same order of magnitude or faster than those for endocytosis, hydrolysis, or protein-mediated transfer, the spontaneous transfer should enter into any scheme in which the rate of turnover of a lipid is evaluated. In human plasma lipoproteins, the halftimes for the turnover of HDL, LDL, and VLDL are approximately 5, 3, and 0.2 days (Schaefer & Ordovas, 1986; Shepherd et al., 1982; Kesaniemi et al., 1982). With eq 6 and the data of Tables 1–3, the calculated halftimes for the transfer of both PDhPC and POPC from HDL, LDL, and VLDL are 4.7, 10.5, and 26 h, respectively. If we consider that measured halftimes for PDhPC are 3.8 times longer than the corresponding calculated times for this lipid, the respective halftimes are 18, 40, and 100 h. Thus, spontaneous transfer is probably an important consideration for HDL and LDL lipid turnover but not for VLDL turnover. The importance of spontaneous transfer rates relative to those for lipolysis and protein-mediated transfer are not known since the molecular specificity of these processes is poorly defined.

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Reversible Inhibition by 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic Acid of the Plasma Membrane (Ca^{2+}+Mg^{2+})ATPase from Kidney Proximal Tubules†

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ABSTRACT: Calcium accumulation by purified vesicles derived from basolateral membranes of kidney proximal tubules was reversibly inhibited by micromolar concentrations of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of anion transport. The inhibitory effect of this compound on Ca^{2+} uptake cannot be attributed solely to the inhibition of anion transport: (Ca^{2+}+Mg^{2+})ATPase activity of the solubilized enzyme was also impaired by DIDS, indicating a direct effect on the calcium pump. The concentrations needed to attain half-maximal inhibition were 20 and 63 μM for (Ca^{2+}+Mg^{2+})ATPase and ATP-dependent Ca^{2+} transport, respectively. The rate constant of EGTA-induced Ca^{2+} efflux from preloaded vesicles was not affected by DIDS, indicating that this compound does not increase the permeability of the membrane vesicles to Ca^{2+}. In the presence of DIDS, the effects of the physiological ligands Ca^{2+}, Mg^{2+}, and ATP on (Ca^{2+}+Mg^{2+})ATPase activity were modified. The Ca^{2+} concentration that inhibited (Ca^{2+}+Mg^{2+})ATPase activity in the low-affinity range decreased from 91 to 40 μM, but DIDS had no effect on the K_m for Ca^{2+} in the high-affinity, stimulatory range. Free Mg^{2+} activated (Ca^{2+}+Mg^{2+})ATPase activity at a low Ca^{2+} concentration, and DIDS impaired this stimulation in a noncompetitive fashion. The inhibition by DIDS was eliminated when the free ATP concentration of the medium was raised from 0.3 to 8 mM, possibly due to an increase in the turnover of the enzyme caused by free ATP accelerating the $E_2 \rightarrow E_1$ transition, and leading to a decrease in the proportion of E_2 forms under steady-state conditions. Alkaline pH totally abolished the inhibition of the (Ca^{2+}+Mg^{2+})ATPase activity by DIDS, with a half-maximal effect at pH 8.3. Finally, the synthesis of ATP catalyzed by soluble enzyme, measured in the same conditions as those of ATP hydrolysis, was insensitive to DIDS up to 100 μM, indicating that this compound acts primarily in the forward direction of the catalytic cycle. Taken as a whole, these results indicate that DIDS interacts with the enzyme in the E_2 conformation, probably slowing the rate of the $E_2 \rightarrow E_1$ transition in forward cycles.

Calcium transport across the basolateral membranes of proximal tubule cells is mediated, in part, by an ATPase that is stimulated by micromolar Ca^{2+} concentrations in the presence of Mg^{2+} (Gmaj et al., 1979, 1982; De Smedt et al., 1981; Vieyra et al., 1986). Although this Ca^{2+} pumping mechanism appears to be important in regulation of intracellular Ca^{2+} concentration, little is known about the catalytic properties of the enzyme. Evidence has been presented (Gmaj et al., 1983; Vieyra et al., 1986) showing that the renal (Ca^{2+}+Mg^{2+})ATPase belongs to the P-ATPase class (Pedersen & Carafoli, 1987), passing through two principal conformational states (E_1 and E_2) during its catalytic cyclic (Figure 1).

In the erythrocyte (Waisman et al., 1981) and in the sarcoplasmic reticulum (Campbell & MacLennan, 1980), the

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