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MULTIPLE FORMS OF 5′-NUCLEOTIDASE FROM LACTATING RAT MAMMARY GLAND RESULTING FROM THE ASSOCIATION OF THE ENZYME WITH DIFFERENT MEMBRANE FRACTIONS

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Summary

Microsomes isolated from rat mammary gland can be separated into two fractions by flotation on a discontinuous sucrose density gradient. The lighter fraction (F_1) exhibits by Lineweaver-Burk analysis two forms of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5), a low K_m form (K_m, 22 μM) and a high K_m for (K_m, 133 μM). The heavier fraction (F_2) shows only the low K_m form. Digitonin treatment of F_1 followed by reflotation permits an additional fractionation (Huggins, J.W. and Carraway, K.L. (1976) J. Supramol. Struct. 5, 59—63). The digitonin-shifted heavier fraction (F_1DF_3) is enriched in plasma membrane markers, is depleted of galactosyltransferase and contains the high K_m form of the nucleotidase. The unshifted fraction (F_1DF_3) is enriched in galactosyltransferase, depleted in plasma membrane markers and contains the low K_m form of the nucleotidase. Digitonin treatment and reflotation of F_2 permits isolation of a density-shifted fraction (F_2DF_3) which is enriched in plasma membrane markers and galactosyltransferase and which contains the unchanged low K_m form of the nucleotidase. Solubilization of either F_1 or F_1DF_3 with deoxycholate or Triton X-100 results in conversion of essentially all of the nucleotidase to a low K_m form.

Arrhenius plots of all forms of the enzyme (high K_m, low K_m, digitonin treated, solubilized) exhibit a temperature transition in the 15—20°C range. In addition membranes exhibiting high K_m forms have a transition near 35°C which is abolished upon treatment with detergents. Inhibition of the low K_m form of nucleotidase by concanavalin A is highly cooperative (Hill coefficient, 1.9—2.4). The high K_m form shows less cooperativity (Hill coefficient, 1.4) in

untreated membranes, but a pronounced increase in cooperativity is observed upon solubilization in detergents.

We propose that the two enzyme forms are best explained by differences in the association of a single type of enzyme molecule with the different types of membranes. According to this proposal specific membrane enzyme-protein or enzyme-lipid interactions place restrictions on the nucleotidase which result in the higher K_m, decreased cooperativity and new thermal transition observed for the enzyme of the F_1 and F_1DF_3 fraction.

Introduction

Although the 5′-nucleotidase (5′-ribonucleotidase phosphohydrolase, EC 3.1.3.5) of mammalian cells has received a considerable amount of attention in recent years, its function is still unclear [1]. The enzyme is localized predominantly at the cell surface in a number of cell types [2—7]. Although it is usually treated as a single species in assessing membrane isolation and purification, there is a considerable amount of disparity in the properties of the enzyme from different sources [8]. Evidence for multiple forms of the enzyme has been presented in the case of the bovine milk fat globule membrane [9].

Recently we have demonstrated that 5′-nucleotidase can be used as a reporter group to monitor changes which occur at the cell surface which result from cellular perturbations [10]. For example, the treatment of intact 13762 mammary ascites tumor cells with colchicine, cytochalasins, dibucaine or the ionophore A23187 in the presence of Ca^{2+} causes an increase in the cooperativity observed for the inhibition of the ecto-nucleotidase by the plant lectin concanavalin A (Ref. 10 and unpublished data). The cooperativity change appears to be related to the mode of association of the enzyme with the membrane. Extraction of isolated ascites plasma membranes under conditions that remove substantial amounts of cytoskeletal proteins (e.g. actin, α-actinin and actin binding protein) [11] causes the inhibition of the membrane nucleotidase by concanavalin A to become cooperative [7].

Such results indicate a need to understand the association of nucleotidase with the membrane and the influence of that association on the properties of the enzyme. Toward this end we have studied a different system, plasma membranes isolated from lactating rat mammary gland. In previous studies on isolation and characterization of plasma membranes from rat mammary gland we noted variations in the kinetics of the nucleotidase depending on the membrane fraction and assay techniques used (Carraway, C.A.C., unpublished data). We now report further analysis, which has shown the presence of two enzyme forms, the apparent K_m values of which are separated about five-fold. The two forms segregate with different membrane fractions which can be separated by density perturbation techniques. The two forms also differ in the cooperativity of a temperature transition of the activity at about 35°C. All of these differences can be eliminated by solubilizing the membranes in deoxycholate or Triton X-100, suggesting that they are related to the mode of association of the enzyme with the membrane.

A preliminary account of a part of this work was presented previously [12].

Experimental procedures

Materials. Partially purified rat mammary plasma membranes (F_1 and F_2) were obtained as previously described [13,14], and subjected to further fractionation by the digitonin shift procedure to yield a highly purified plasma membrane fraction and a Golgi fraction [14]. F_1 and F_1DF_1 fractions were obtained from the 0.9%-32% sucrose interface, F_2 from the 32%-36% sucrose interface and F_1DF_3 and F_2DF_3 from the 36%-40% interface (Ref. 14 and unpublished data). Concanavalin A, enzymes, substrates and deoxycholate were from Sigma Chemicals.

Analytical procedures. For the assay of 5'-nucleotidase, membranes or solubilized fractions at an appropriate concentration were suspended in assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.0) and incubated for 10 min at 37°C. Subsequently β-glycerophosphate was added to 5 mM prior to initiation of the reaction. The reaction was started by adding AMP (containing 1—10 μCi/ml of [^{32}P]AMP) to make a total assay volume of 0.4 ml. The reaction was run at the appropriate temperature and terminated by adding 1 ml of ice-cold 10% (w/v) trichloroacetic acid solution containing 10% (w/v) acid-washed charcoal. The assay tubes were then centrifuged at 5000 × *g* for 15 min to sediment the charcoal, and a 500 μl aliquot of the supernatant in Aquasol-2 was counted in a Packard Tri-Carb Model 3320 Liquid Scintillation Counter. Blanks were made in which no enzymes were added.

Protein was determined by the method of Lowry et al. [15].

Concanavalin A inhibition experiments were performed as previously described [7,16].

Results

Multiple enzyme forms in purified mammary membrane fractions

Plasma membrane-enriched material is obtained in two fractions (F_1 and F_2) after flotation of a microsomal preparation on a discontinous sucrose density gradient [13]. When the partially purified light rat mammary plasma membrane fraction (F_1) was used, complex kinetic behavior was observed for the nucleotidase. Lineweaver-Burk plots showed two distinct straight lines (Fig. 1), suggesting the presence of two forms of the enzyme. When the heavy plasma membrane fraction (F_2) was examined, an essentially hyperbolic response was observed for the substrate concentration dependence of the nucleotidase velocity. The Lineweaver-Burk analysis (Fig. 2) gave a K_m of 27 μM, essentially identical to the low K_m form of the enzyme in F_1. However, it should be noted that the Lineweaver-Burk plot shows a slight 'tailing' at the high substrate end, suggesting the presence of a small amount of enzyme with a higher K_m. This is not too surprising, since the F_2 fraction, upon recentrifugation, will reequilibrate to yield some F_1 (unpublished data).

The results above suggest that there are two forms of the nucleotidase present in the partially purified F_1 fraction. Since this fraction contains two different types of membranes, which can be separated by digitonin treatment and flotation on a density gradient [19], we examined the separated subfractions (F_1DF_1 and D_1DF_3) to determine whether the two enzymes forms are

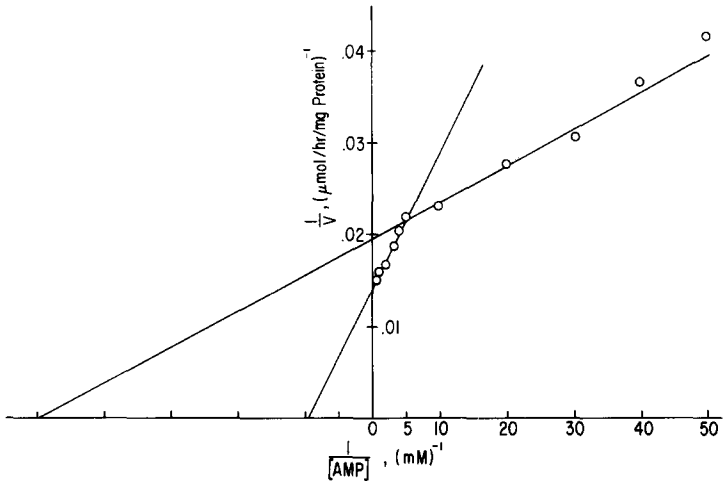

Fig. 1. Lineweaver-Burk plot of F_1 membrane fraction 5'-nucleotidase kinetics. Membranes were prepared, incubated and assayed as in Experimental procedures. Membrane protein concentration was 22 μg/assay.

segregated in these two types of membranes. The digitonin treatment of the F_1 fraction did not significantly alter the kinetic behavior of the enzyme (Table I).

When the purified fractions F_1DF_1 and F_1DF_3 are characterized separately, they show clearly different kinetic plots, indicating that each form of the enzyme is present in a different type of membrane. The low K_m form is associated with the putative Golgi fragments (F_1DF_1), while the high K_m form is associated with putative plasma membrane (F_1DF_3) (Table I). Treatment of F_2 with digitonin also yields a fraction (F_2DF_3) with an increased density, whose nucleotidase has a K_m essentially unchanged from the parent F_2 fraction (Table I).

Effects of membrane solubilization on the enzyme forms

There are two likely explanations for the forms observed in the different

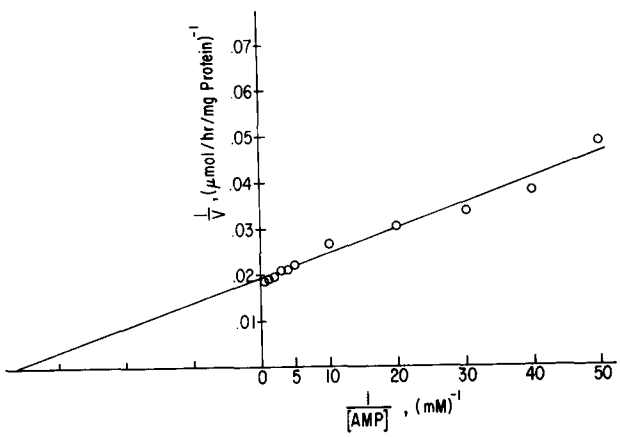

Fig. 2. Lineweaver-Burk plot of F_2 membrane fraction 5'-nucleotidase kinetics.

TABLE I

K_m VALUES FOR 5′-NUCLEOTIDASE OF MEMBRANE AND SOLUBILIZED FRACTIONS OF NORMAL RAT MAMMARY GLAND

Membrane fraction	K_m (μM)
F_1 high K_m	130
F_1 low K_m	22
F_1 + deoxycholate	28
F_1 + Triton X-100	23
F_1 + digitonin:	
High K_m	120
Low K_m	18
F_1DF_1	28
F_1DF_1 + Triton X-100	21
F_1DF_3	120
F_1DF_3 + deoxycholate	37
F_1DF_3 + Triton X-100	34
F_2	27
F_2DF_3	26

membrane fractions. (1) There are two different types of 5′-nucleotidase molecules with different properties. (2) There is one type of nucleotidase molecule, but its properties vary with its mode of association with the membrane and with the properties of the membrane. If the latter is true, it should be possible to eliminate the differences between the forms by disrupting the membrane structure, e.g. by solubilizing the enzyme. Fig. 3 shows the 5′-nucleotidase activity of the F_1 fraction solubilized by deoxycholate. More than 80% of the enzyme was soluble, and the curves for solubilization of the two enzyme forms, as assayed at 0.1 mM (Fig. 3) and 1.0 mM (not shown) substrate, were essentially identical. Kinetic studies of the soluble enzyme indicated that the low K_m enzyme was by far the predominant species (Table I).

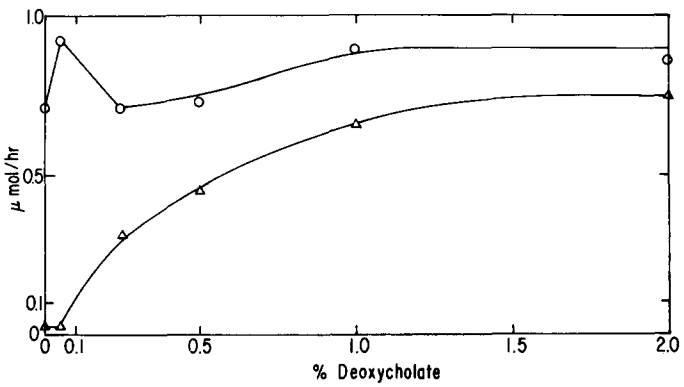

Fig. 3. Solubilization of 5′-nucleotidase activity with deoxycholate. F_1 membranes were suspended in 50 mM Tris (pH 8.0) containing various concentrations of deoxycholate. After a 1 h incubation at 37°C, the suspensions were centrifuged at 40 000 × *g* for 2.5 h. The supernate was assayed for 5′-nucleotidase activity in the presence of 0.1 mM AMP (containing 1—5 μCi/ml [^{32}P]AMP). This is referred to as the solubilized activity (△———△). The total activity (○———○) represents the activity present before the centrifugation.

A dramatic reduction in K_m was observed when purified F_1DF_3 was treated with deoxycholate (Table I). This behavior is consistent with the premise that the properties of the enzyme in the F_1DF_3 fraction are altered by its association with the membrane, since these properties more closely resemble the low K_m form when the membrane is solubilized.

Similar results were obtained when the non-ionic detergent Triton X-100 was used. The K_m values are not significantly different from those found with deoxycholate (Table I). When membranes containing only the low K_m form of nucleotidase (e.g. F_1DF_1) are solubilized in either deoxycholate or Triton X-100, the K_m values for the enzyme are not substantially changed (Table I).

Temperature dependence of the nucleotidase activity

As a further indication of the influence of the membrane-enzyme association the temperature dependence of the nucleotidase in the various membrane fractions was examined. Arrhenius plots for the F_1 fraction assayed with 1.0 mM AMP showed two breaks (Fig. 4). The F_1 fraction assayed at 0.1 mM AMP and

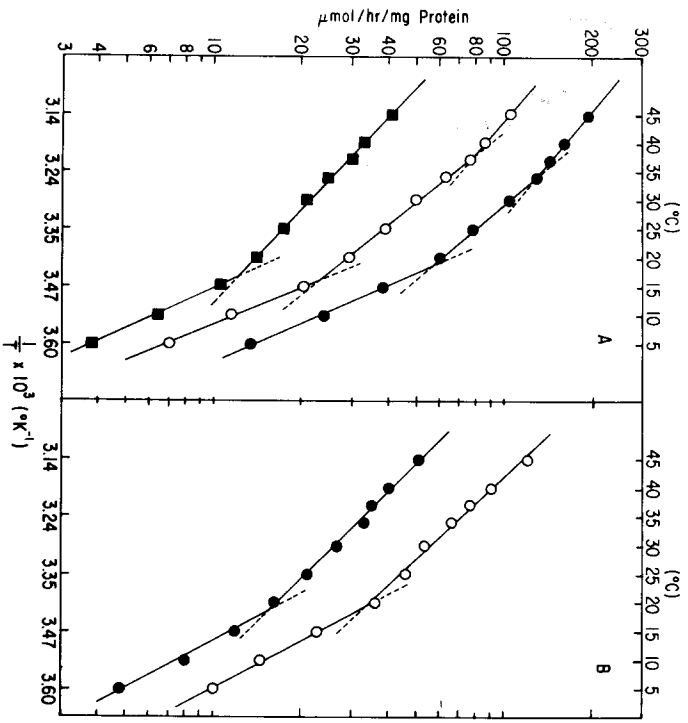

Fig. 4. Arrhenius plots for mammary membranes and Triton-solubilized fractions. (A) Membranes were incubated at the appropriate temperature in assay media for 10 min prior to assaying at the same temperature. F_1DF_3 membranes (●) and F_1 membranes (○) were assayed with 1.0 mM ATP, while F_2 membranes (■) were assayed with 0.1 mM AMP. (B) Membranes were suspended in 10 mM Tris (pH 8.0) containing 1% Triton X-100 and incubated for 1 h at 37°C. The solubilized membranes were then incubated at the appropriate temperature in assay media for 10 min prior to assaying at the same temperature. F_1 Triton-solubilized membranes (○) were assayed with 1.0 mM AMP, and F_2 Triton-solubilized membranes (●) were assayed with 0.1 mM AMP.

TABLE II

TRANSITION TEMPERATURES AND ACTIVATION ENERGIES FOR NUCLEOTIDASE IN VARIOUS MEMBRANE AND SOLUBILIZED FRACTIONS

All values reported are averages of two or three separate determinations on different membrane preparations with standard deviations of less than 10% of the reported values. The blanks indicate no treatment or a failure to observe a corresponding value for that sample.

Fraction	Treatment	AMP (mM)	Activation energy (kcal/mol)			Transition temperature (°C)	
			E_{a1}	E_{a2}	E_{a3}	T_1	T_2
F_1	—	1.0	17.8	10.5	7.4	15.3	36.7
F_1	—	0.1	13.4	7.4	—	18.0	—
F_2	—	0.1	14.3	8.0	—	18.2	—
F_1	digitonin	0.1	14.8	7.3	—	18.3	—
F_1	digitonin	1.0	17.7	9.2	6.8	17.6	37.7
F_1DF_3	—	1.0	16.9	10.1	7.3	18.2	34.4
F_1	deoxycholate	0.1	14.0	7.7	—	19.2	—
F_1	deoxycholate	1.0	14.4	9.0	—	19.2	—
F_2	deoxycholate	0.1	14.0	8.6	—	19.1	—
F_1	Triton X-100	1.0	13.6	7.6	—	20.7	—
F_1	Triton X-100	0.1	13.2	7.1	—	19.2	—
F_2	Triton X-100	0.1	13.7	8.0	—	18.8	—

the F_2 fraction gave only one break (Table II). Treatment of F_1 with the digitonin did not alter this behavior, except for a slight shift in the lower transition temperature. Purified F_1DF_3 also showed both transitions (Fig. 4). When F_1 is treated with deoxycholate or Triton X-100, the high temperature transition observed in the presence of 1.0 mM AMP is lost (Fig. 4 and Table II).

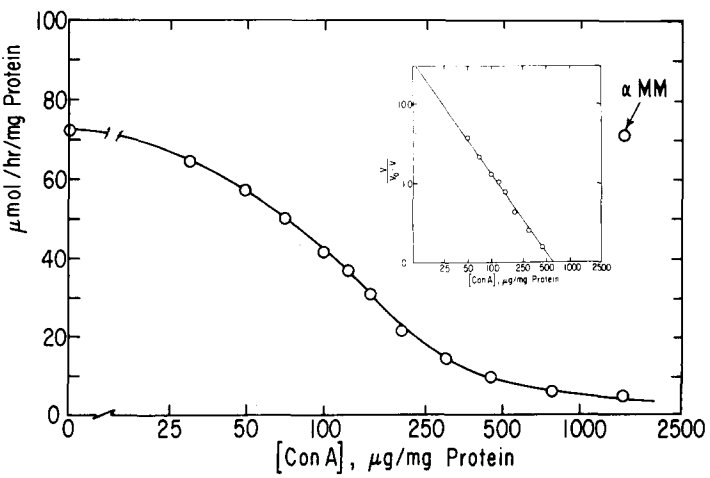

Fig. 5. Concentration dependence of concanavalin A inhibition of F_1 membrane 5 -nucleotidase assayed with 1 mM AMP. Membranes, prepared as described in Experimental procedures and Fig. 1, were incubated 10 min with concanavalin A before assaying with 1 mM AMP (containing 1—5 μCi/ml [^{32}P]-AMP). 50 mM α-methylmannoside (α-MM) was added to a tube containing the highest concanavalin A concentration to show reversibility of the reaction. The α-methylmannoside was added after incubation with concanavalin A and the membranes incubated an addition 10 min before assaying. The inset shows a Hill plot for concanavalin A inhibition of F_1 membrane 5'-nucleotidase (n, 1.4).

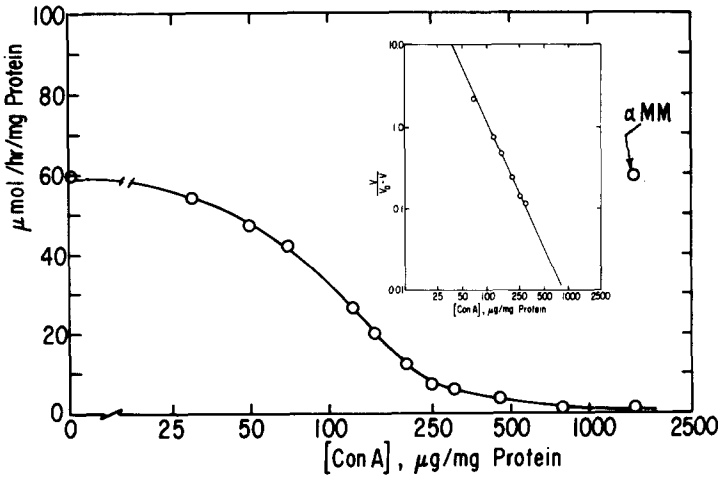

Fig. 6. Concentration dependence of concanavalin A inhibition of F_1 membrane 5'-nucleotidase assayed with 0.1 mM AMP. Membranes were prepared, incubated and assayed as in Fig. 3 except that 0.1 mM AMP (containing 1—5 µCi/ml [^{32}P]AMP) was used. The inset shows a Hill plot for concanavalin A inhibition of F_1 membrane 5'-nucleotidase in the presence of 0.1 mM AMP (n, 2.2).

There is essentially no change in the low temperature transition resulting from detergent treatments. Thus the loss of the high temperature transition on detergent treatment is correlated with the change in K_m previously noted. There is no obvious relationship between the low temperature transition and K_m. Data for the transition temperatures and activation energies of the various membrane and solubilized species (Table II) show the similarity of the solubilized forms to the form observed in membranes exhibiting a low K_m enzyme.

Cooperativity of the concanavalin A inhibition

Treatment of F_1 fractions with concanavalin A caused essentially complete inhibition of either the high K_m (Fig. 5) or low K_m (Fig. 6) enzyme. Both were readily reversible by α-methylmannoside, indicating a specific interaction of the concanavalin A with carbohydrate. However, the degree of cooperativity was different for the two. A Hill coefficient of 1.4 was seen with the high K_m

TABLE III

COOPERATIVITY IN THE CONCANAVALIN A INHIBITION OF NORMAL RAT MAMMARY MEMBRANE 5'-NUCLEOTIDASE

Membrane fraction	Treatment	AMP (mM)	Hill coefficient
F_1	—	0.1	2.3
F_1	—	1.0	1.4
F_2	—	0.1	1.9
F_1DF_3	—	1.0	1.4
F_1DF_3	deoxycholate	0.1	2.1
F_1DF_3	deoxycholate	1.0	2.1
F_1	deoxycholate	1.0	2.2
F_1	Triton X-100	1.0	2.1

enzyme form, whereas a Hill coefficient of 2.2 was observed for the low K_m form. Purified F_1DF_3, which shows a high K_m enzyme, also exhibits decreased cooperativity (Table III).

When membranes containing the high K_m enzyme were solubilized in deoxycholate, there was a pronounced increase in cooperativity (Table III) along with the decrease in K_m previously described. In addition the F_2 membrane fraction, which has only the low K_m enzyme, exhibits cooperativity for the concanavalin A inhibition (Table III).

Discussion

Rat mammary gland microsomes can be separated by flotation into two fractions (F_1 and F_2) which contain plasma membrane material [13]. Both are enriched in 5′-nucleotidase, ($Na^+ + K^+$)-ATPase, cholesterol, sialic acid and galactosyltransferase. The last observation suggests the presence of Golgi in the preparations, since galactosyltransferase is enriched in the Golgi apparatus. If F_1 is treated with digitonin and refloated on a gradient with the same composition, a portion of the membranes moves to a higher density. This material (F_1DF_3) is highly enriched in nucleotidase, sialic acid and cholesterol but has no galactosyltransferase [14]. The portion of the digitonin-treated fraction which retains the original density upon recentrifugation, called F_1DF_1, is enriched in galactosyltransferase but depleted in nucleotidase, cholesterol and sialic acid. The F_1DF_3 and F_1DF_1 fraction are therefore considered to be purified plasma membranes and Golgi fragments, respectively. If F_2 is treated with digitonin, a portion of the sample migrates to a higher density. This F_2DF_3 is enriched in nucleotidase, sialic acid, cholesterol and galactosyltransferase, suggesting that it is a plasma membrane containing galactosyltransferase (unpublished data). This proposal is supported by the similarity of sialoglyprotein profiles for F_2DF_3 and the milk fat globule membrane of rat milk (unpublished data).

Kinetics of the nucleotidase in the various membrane fractions indicate the presence of two enzyme forms in F_1. These forms segregate with the F_1DF_1 and F_1DF_3 fractions. Thus a question arises as to the nature of the differences between the two forms. One obvious possibility is that they represent two different enzyme molecules (isoenzymes) which are separately synthesized and incorporated into the different types of membranes. We feel this is unlikely because the high K_m form of the enzyme can be converted to a form with properties closely resembling the low K_m form by treatment with detergents. The conversion can be accomplished by two different detergents with different solubilizing properties and different effects on membrane enzymes. In addition three different properties of the enzyme are coordinately altered.

In view of these results a more likely explanation is that there is one type of enzyme whose properties are dependent on its association with the membrane. According to this proposal the increased K_m and decreased cooperativity would be a result of restrictions placed on the enzyme by its association with other membrane components. This behavior would correspond with the observation of a new transition in the Arrhenius plots, indicative of the presence of another form of the enzyme. This transition is eliminated by detergent treatments, sug-

gesting that it is dependent upon interactions that are disrupted by the detergents. The transition near 20°C is not influenced by detergents and shows no correspondence to changes in the other enzyme properties. These results suggest that this transition is probably a function of a change in the enzyme molecule itself, although other tightly bound species could be involved. The fact that the transition near 20°C is not substantially altered by detergent treatments is another indication that the detergents are not substantially altering the structure of the enzyme molecule, even though they are changing the K_m and the response to concanavalin A.

Thus we feel there is no necessity for postulating the occurrence of isoenzymes to explain the differences between enzymes of F_1DF_3 and F_1DF_1. However, this should not be taken to indicate that there are necessarily no molecular differences between the two enzyme forms. Since the nucleotidase is a glycoprotein, it is quite possible that the two forms of the enzyme, which are found in different types of membranes, could have variations in carbohydrate content which would give them different physical properties without significantly affecting their K_m values or the cooperativity parameter.

The molecular basis for the difference in the properties of the two forms is still unclear. It is interesting to note that the two purified membrane fractions (F_1DF_3 and F_2DF_3) which have characteristics of plasma membranes (high nucleotidase, cholesterol and sialic acid) have different forms of the enzyme. The enzyme and density differences of these fractions indicate that they must have differences in protein or lipid compositions. Preliminary experiments using fluorescent probe techniques do not show prominent differences in microviscosities which can be related to the enzyme behavior (Doss, R.C. and Smith, W.B., unpublished data). This is supported by observations on membrane cholesterol, which is a primary modulator of membrane fluidity [17]. Cholesterol values are high for F_1DF_3 and F_2DF_3, which have different nucleotidase forms. In addition F_1DF_1, which has low cholesterol, and F_2DF_3, which has high cholesterol, apparently have the same enzyme form. Thus there appears to be no relationship between cholesterol and the kinetics of the membrane enzyme. These results suggest that the observed behavior of the enzyme in these membranes is dependent on specific enzyme-lipid or enzyme-protein interactions rather than on the bulk membrane lipid phase. Nucleotidase isolated from rat liver shows a specific association with sphingomyelin [18]. Whether such an association exists in the mammary membranes requires further investigation.

In previous studies we have shown that the cooperativity of the nucleotidase inhibition by concanavalin A is dependent on the 'state' of the membrane [7]. We postulated that the cooperativity was dependent on the multivalency of the concanvalin A and mobility of the enzyme in the membrane [16]. The present results are consistent with this hypothesis, if it is also assumed that decreased K_m and increased cooperativity correlate with a release of restraints in the membrane imposed by specific lipid-enzyme or protein-enzyme interactions, as suggested earlier. Further investigations by membrane modification and reconstitution techniques should aid in defining these parameters and increase our understanding of the behavior of the nucleotidase and membrane enzymes in general.

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