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ISOLATION AND PARTIAL PURIFICATION OF DICARBOXYLIC ACID BINDING PROTEIN FROM LUMINAL-MEMBRANE VESICLES OF RABBIT KIDNEY CORTEX

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A specific dicarboxylic acid binding protein was isolated by solubilizing highly purified renal luminal-membrane vesicles with the non-ionic detergent $C_{12}E_8$, followed by affinity chromatographic procedures. SDS-polyacrylamide gel electrophoresis of the samples containing dicarboxylic acid binding protein showed a single sharp band of an apparent molecular weight of 50000. After treatment with mercaptoethanol the protein was split in two subunits of apparent molecular weights of 35000 and 15000. By analytical ultracentrifugation the minimal molecular weight of the dicarboxylic acid binding protein preparation was calculated to be 54000. Binding of the radioactive succinate and L-malate to the dicarboxylic acid binding protein preparation as studied by equilibrium dialysis showed saturation phenomenon and was specifically inhibited by addition of D-malate. The dissociation constants for succinate (0.18 mM) and L-malate (0.33 mM) calculated from the binding data agree extremely well with the apparent K_m values for these organic acids found in transport studies utilizing intact luminal-membrane vesicles.

Isolation of transport proteins is fundamental in the biochemical study of these proteins, but has only been achieved in relatively few instances. Most previous attempts to isolate transport proteins for organic compounds in mammalian systems have centered around the sodium D-glucose transporter in various organs of epithelial origin such as kidney [1–4] and intestine [5]. Definitive results have been difficult to obtain, primarily because the transport protein is present in very small amounts [6] and due to the fact that treatment with detergents mostly leads to irreversible inactivation of the protein.

It is now well established that luminal membranes of proximal tubule of mammalian kidney possess a Na^+-dicarboxylic acid cotransport system [7–15]. Wright et al. [9] studied the structural specificity of the dicarboxylic acid transport system in luminal-membrane vesicles by determining the relative inhibitory constants of 40 different organic acids on the transport of succinate. They found that the system is highly specific for 4-carbon terminal dicarboxylic acids in the *trans*-configuration, including the major intermediates of the Krebs cycle. Further understanding of the molecular basis of the renal Na^+-dependent dicarboxylic acid transport system, however, largely depends on progress in the purification of the membrane transport protein responsible for it.

We here wish to report a procedure for the isolation of a membrane protein that may be involved in dicarboxylic acid transport which can be prepared from highly purified renal luminal-membrane vesicles by affinity chromatography in relatively large yield and with retention of binding

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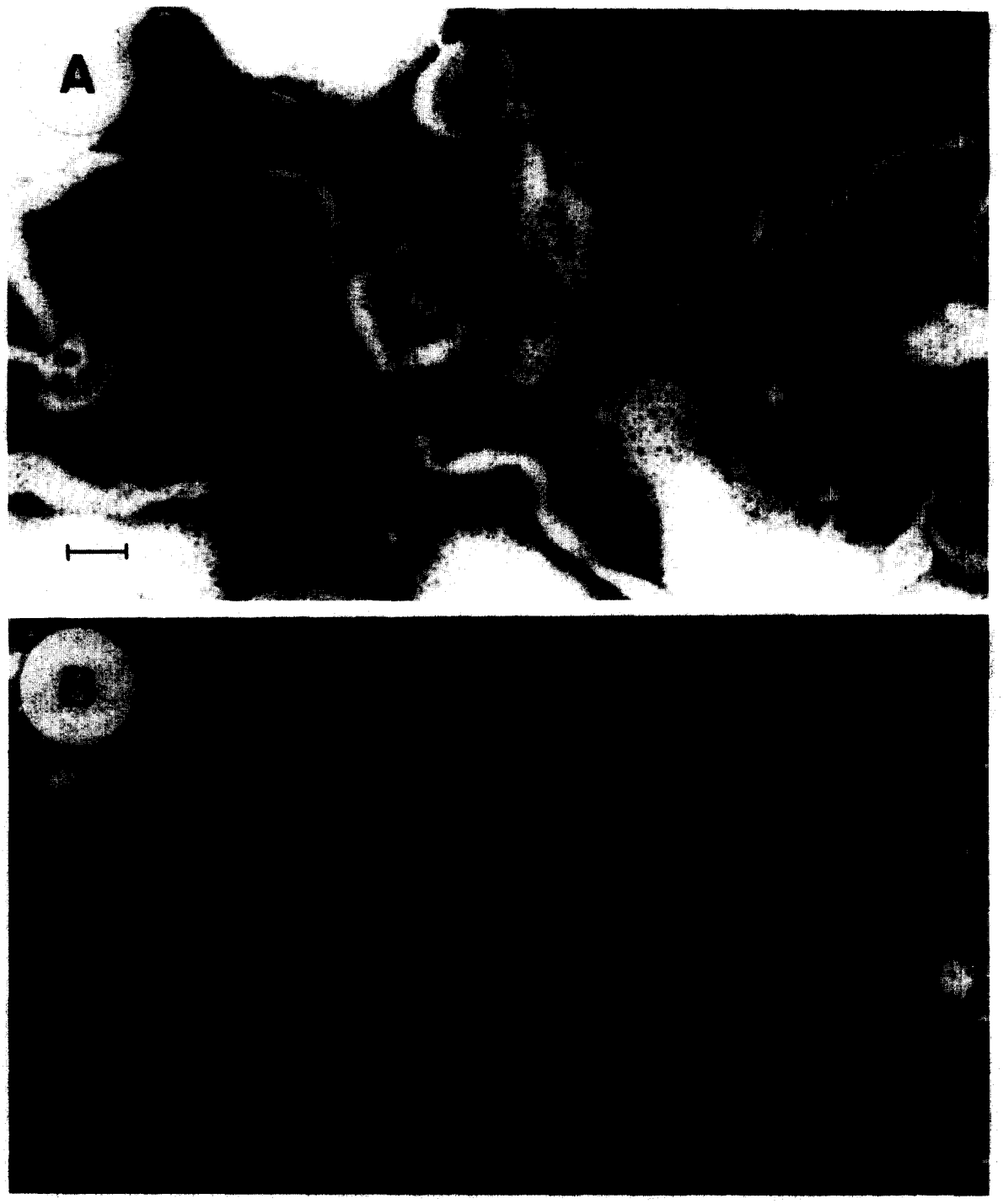


Fig. 1. Electron micrographs. Samples were taken from a vesicle suspension before (A) and after addition of $C_{12}E_8$ (B). A small volume was placed on a grid, negatively stained with 2% phosphotungstic acid (pH 7.2) and examined with a Zeiss 10B electron microscope operating at 60 kV. Magnification, ×96700. Bar, 0.1 μm.

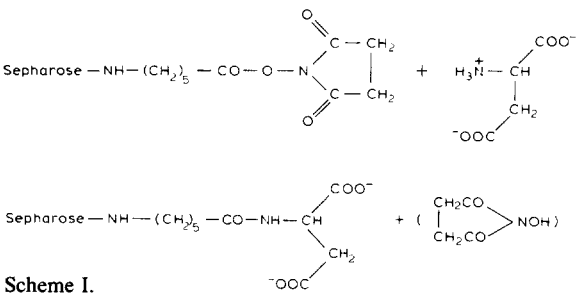
properties. To solubilize the membrane we used the non-ionic detergent $C_{12}E_8$ (octaethyleneglycol mono *n*-dodecyl ether) which previously has been successfully used to solubilize the sarcoplasmic reticulum Ca^{2+}-ATPase from skeletal muscle [16,17] and $(Na^+ + K^+)$-ATPase from rectal gland of *Squalus acanthias* [18,19] and from pig kidney [20] in an active state.

Luminal-membrane vesicles were isolated from rabbit kidney cortex according to the method recently described from this laboratory [15]. All solutions were sterilized before used. The membrane fraction was assayed for various marker enzymes also as previously described [21]. The results of these measurements showed that the luminal-membrane vesicles thus obtained were completely free of cytosolic and mitochondrial proteins (the enzyme activities of neither lactate dehydrogenase nor succinate dehydrogenase were detectable) and largely free of basolateral membranes.

The characteristics of renal uptake of succinate and L-malate by luminal-membrane vesicles were studied by the spectrophotometric method recently described from this laboratory [14].

Highly purified luminal-membrane vesicles were dissolved in the medium containing 25 mM Hepes/Tris (pH = 7.5), 0.02% NaN_3, 150 mM NaCl and non-ionic detergent $C_{12}E_8$ in desirable concentration that gives rise to a final protein: $C_{12}E_8$ ratio of 1 : 3. Figs. 1A and 1B show electron micrograph of negatively stained luminal-membrane vesicles before and after solubilization with $C_{12}E_8$, respectively. It appears from Fig. 1A that the luminal-membrane vesicles are covered with a coat of small particles on their outer surface, indicating right-side-out and intact vesicles [22]. Fig. 1B shows that the luminal-membrane vesicles are completely solubilized after addition of $C_{12}E_8$. Small particles are scattered all over the field and pieces of recognizable cellular membranes were not observed. The completely solubilized luminal-membrane protein fraction was applied on the affinity column and chromatographed as mentioned below.

L-Aspartate-Sepharose column was prepared by mixing activated CH-Sepharose 4B with L-aspartate according to the reaction sequence given in Scheme I. Activated CH-Sepharose 4B (4 g) was



Scheme I.

first washed with ice-cold 600 ml HCl (1 mM) and then the gel was suspended in 18 ml 0.1 M $NaHCO_3$ buffer (pH 8.0) which contained 20 mg L-aspartate. The gel was carefully mixed by rotating for 1 h at 25 °C. After the completion of the above-mentioned reaction the gel was washed alternatively with a solution containing 0.05 M Tris (pH 8.5) and 0.5 M NaCl and with a solution containing 0.1 M sodium acetate (pH 4.0) and 0.5 M NaCl for 1 h. In a series of control experiments the degree of L-aspartate coupling to Sepharose was determined by using radioactive L-[^{14}C]aspartate. The degree of coupling varied from 10 to 15% based on added L-aspartate.

The ultimate aim of performing affinity chromatography experiments was to prepare a gel column containing a free dicarboxylic anion arm, which may specifically bind the dissolved membrane protein responsible for the transport of dicarboxylic acid by luminal-membrane vesicles. A portion of luminal-membrane vesicles solubilized in $C_{12}E_8$ (5 ml containing 50 mg protein) were applied to the affinity column and eluted with 25 mM Hepes-Tris buffer (pH 7.5) containing 150 mM NaCl, 0.02% NaN_3, and 0.02% $C_{12}E_8$ (w/v). The critical micellar concentration of $C_{12}E_8$ at room temperature is 0.05 mg/ml [23], so that the concentration of $C_{12}E_8$ in the eluate is much higher than that needed to maintain the proteins in a completely solubilized state. Fig. 2 shows the results obtained by using affinity chromatographic technique. First a large protein peak was eluted (not shown) corresponding to void volume of the column. The chromatographic column was then washed with 90 ml of the same buffer solution. After that the composition of elution buffer was changed to include 100 mM succinate in order to elute binding protein from the column. As can be

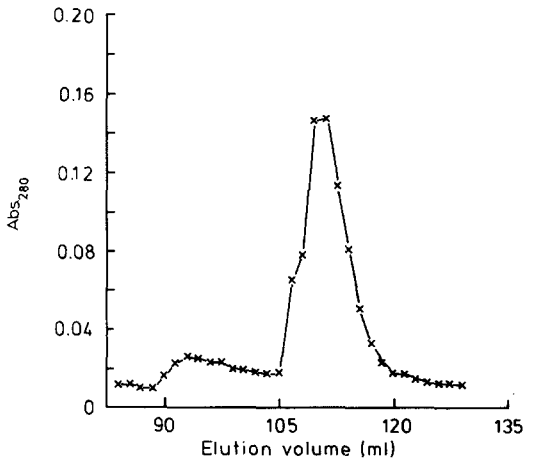


Fig. 2. Elution pattern of dicarboxylic acid binding protein obtained by the use of affinity chromatography of solubilized luminal-membrane vesicles by non-ionic detergent ($C_{12}E_8$).

seen from Fig. 2 a protein peak is obtained in the elution volume range of 105–120 ml. The total amount of protein as determined by the method of Lowry et al. [24] and modified by Peterson [25] was 0.3 mg (0.6% of total membrane proteins). The apparent high extinction at 280 nm observed in Fig. 2 is due to turbidity of the solution. In a series of control experiments elution buffer containing 100 mM succinate was replaced by buffer containing D-glucose or L-phenylalanine, the compounds which are shown to be transported into luminal-membrane vesicles [26,27]. In these experiments no protein peak was observed, strongly indicating that the protein eluted in the elution volume range of 105–120 ml is specific dicarboxylic acid binding protein. The protein containing samples were pooled and succinate was separated by passing the protein-succinate mixture through a Sephadex G-25 column by using succinate free Hepes-Tris buffer as eluant. The samples were transferred into a cellophane dialysis bag. In order to concentrate the protein solution the dialysis bag was then transferred into a beaker containing saturated solution of polyethylene glycol of a molecular weight of 20000 (PEG-20000) which efficiently absorbs water. In this way the fractions were concentrated to a final protein concentration of 2 mg/ml and stored at −18°C. Electron microscopic examination of the preparation showed only protein particles, but not any lipoprotein vesicles. This protein sample is denoted as dicarboxylic acid binding protein.

Electrophoretic experiments were performed by using 7.5% and 12% acrylamide gel containing 0.2% SDS dissolved in Tris buffer (Fairbank buffer) (pH 7.4) according to the method of Weber and Osborn [28]. The protein bands were made visible by adding Coomassie brilliant blue R-250 dye to the gel. The molecular weight of dicarboxylic acid binding protein preparation was determined both by gel electrophoresis and by analytical centrifugation. Analytical centrifugation experiments were performed by using a Beckman Model E analytical ultracentrifuge, equipped with photoelectric scanner. Molecular weight (M_P) was determined in sedimentation equilibrium experiments from plot of ln C (light absorption of solution of 280 nm) versus r^2 (distance from center of rotation) according to the equation given by Tanford et al. [29]

$$M_P(1 - \phi\bar{\rho}) = M_P(1 - \bar{\nu}\rho) + M_P\delta_{C_{12}E_8}\left(1 - \bar{\nu}_{C_{12}E_8}\rho\right)$$

where $(1 - \phi\bar{\rho})$ is the factor to be employed for conversion of the buoyant weight of the protein-$C_{12}E_8$ particle to protein molecular weight; $\bar{\nu}\rho$ and $\bar{\nu}_{C_{12}E_8}$ are the partial specific volumes of protein and bound non-ionic detergent, respectively; $\delta_{C_{12}E_8}$ is the amount of bound $C_{12}E_8$, expressed on a gram per gram basis. Value of partial specific volume of protein was assumed to be 0.74 ml/g. The partial specific volume of $C_{12}E_8$ was taken from Tanford et al. [30].

Dissociation constants for binding of succinate and L-malate to the dicarboxylic acid binding protein preparation were determined by equilibrium dialysis. Test dialysis bags were filled with 1 ml solution containing dicarboxylic acid binding protein and desired concentrations of radioactive [^{14}C]succinate or L-[^{14}C]malate dissolved in 25 mM Hepes-Tris (pH 7.5) and 150 mM NaCl and the bags were immersed in volumetric flasks containing 5 ml of the same solution, minus protein. The reference dialysis bags contained the same solution, minus protein, and were dialyzed as described above. The concentration of $C_{12}E_8$ both in test and reference bags as well as in dialyzed medium was the same. When equilibrium was reached (after 6 h) the samples were drawn from

the test and reference dialysis medium and their radioactive contents were analyzed by liquid-scintillation counter (Wallac LKB 1210 ultrabeta) using lumagel (Lumac, The Netherlands). In a series of experiments the effect of D-malate on the binding of L-malate to the dicarboxylic acid binding protein preparation was examined. These experiments were carried out as mentioned above except that in this case both the test and reference bags contained 200 μM D-malate, in addition to L-malate. All experiments were performed at 25 °C. Binding of succinate and L-malate to the dicarboxylic acid binding protein preparation was calculated according to the following equation:

$$B = \frac{P \cdot L}{K_d + L}$$

where B indicates the concentration of bound ligand, P is the total protein concentration, L represents the concentration of free ligand and K_d denotes dissociation constant. The curve fitting was based on a statistical procedure as recently described [31], in which iteration program is a least-squares procedure that minimizes the square root of the sum of square deviations in the y-axis direction.

The mixture of solubilized luminal-membrane proteins, which appeared in the void volume as described above, and dicarboxylic acid binding protein samples in duplicate were treated with 2.5% SDS and subjected to electrophoresis, using 7.5% polyacrylamide gel (Fig. 3A). Channels 2 and 4, and 1 and 3 in Fig. 3A depict the electrophoretic pattern of the dicarboxylic acid binding protein preparation and other luminal-membrane proteins, respectively. It is seen that the dicarboxylic acid binding protein preparation contained a single sharp band, and a diffuse band. In order to determine apparent molecular weight of dicarboxylic acid binding protein, duplicate samples of the dicarboxylic acid binding protein preparation (channels 2 and 3 in Fig. 3B) and proteins with known molecular weights (channels 1 and 4 in Fig. 3B) for standard calibration were subjected to electrophoresis, using 12% polyacrylamide gel. The molecular weight of dicarboxylic acid binding protein is assessed by this analysis to be approx. 50 000. Electrophoretic analysis of the dicarboxylic acid binding protein preparation after treatment with mercaptoethanol (Fig. 3C), however, showed two weak, but distinct bands, with apparent

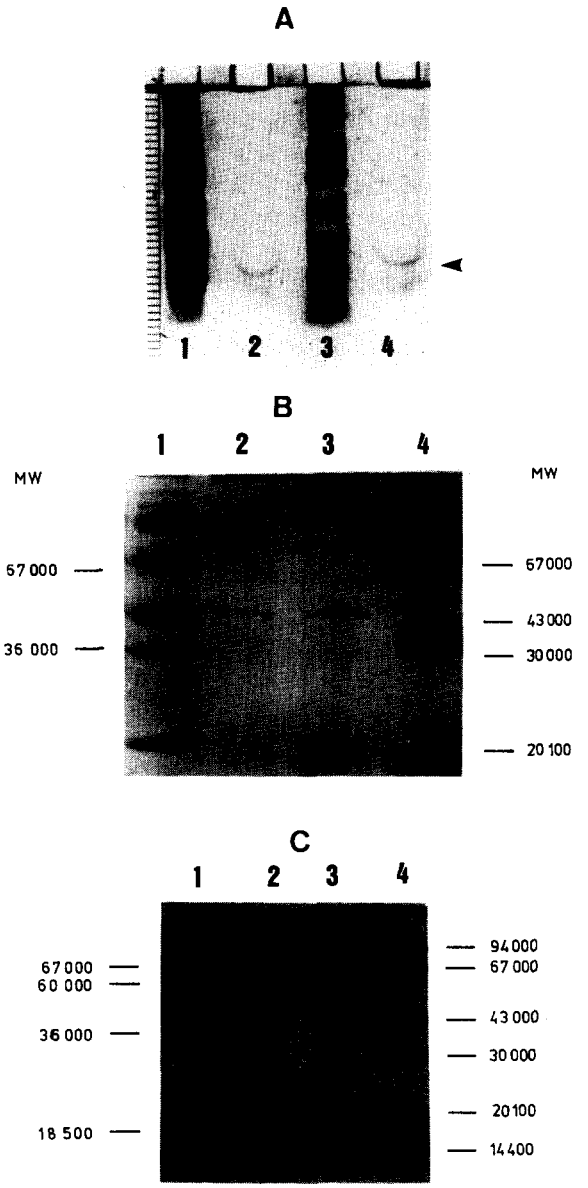


Fig. 3. (A) SDS-polyacrylamide gel electrophoretic pattern of solubilized luminal-membrane proteins appeared in void volume (channels 1 and 3) and the dicarboxylic acid binding protein preparation (channels 2 and 4). (B) Electrophoresis of known molecular weight proteins (channels 1 and 4) and the dicarboxylic acid binding protein preparation (channels 2 and 3). (C) Electrophoretic pattern of the dicarboxylic acid binding protein preparation (channels 2 and 3) after treatment with mercaptoethanol. For detailed description see text.

molecular weights of approx. 35000 and approx. 15000, suggesting that dicarboxylic acid binding protein probably consists of two polypeptide chains linked together with S-S bond. It should be emphasized here that the results shown in Fig. 3 indicate that the dicarboxylic acid binding protein preparation is only partially purified by the analytical procedures used in this paper.

The molecular weight of intact dicarboxylic acid binding protein was further determined by analytical ultracentrifugation. Analysis of the sedimentation equilibrium experimental data showed that the plot of ln c versus r^2 was slightly curved, again indicating heterogeneity of the dicarboxylic acid binding protein preparation. The minimal molecular weight of dicarboxylic acid binding protein calculated from these experiments was 54000.

Fig. 4A shows the characteristics of binding of [^{14}C]succinate to the dicarboxylic acid binding protein preparation. It appears from the figure that the degree of binding of succinate to dicarboxylic acid binding protein increases with increasing concentrations of free ligand and approached saturation at higher concentrations (0.8–1 mM) of succinate. The reciprocal values of the binding data are plotted in the inset of Fig. 4A. From this analysis the dissociation constant (K_d) value was calculated to be 0.18 mM. Fig. 4B shows the absorbance changes induced by addition of increasing concentrations of succinate to intact luminal-membrane vesicles taken from the same preparation that was later used to isolate

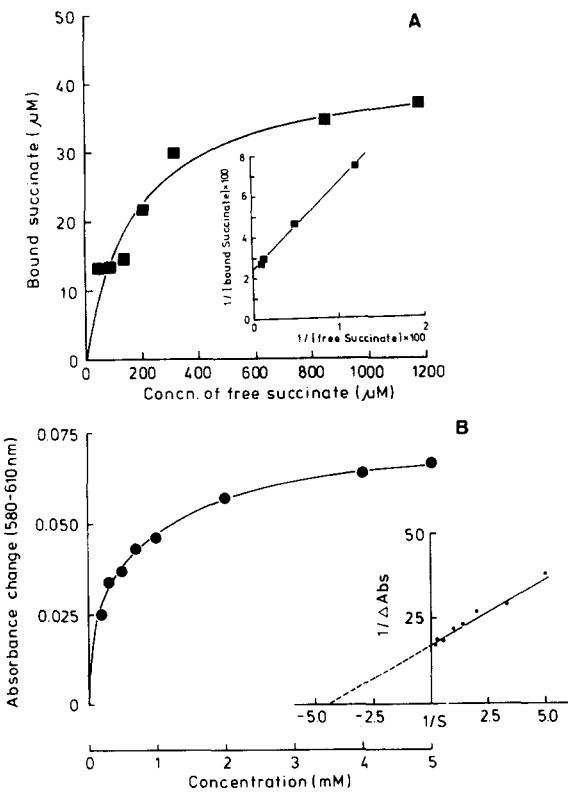


Fig. 4. (A) Binding of succinate to the dicarboxylic acid binding protein preparation. Inset shows a reciprocal plot of the experimental data. (B) Uptake of succinate by intact luminal-membrane vesicles as studied by spectrophotometry. The inset shows a Lineweaver-Burk analysis of the data.

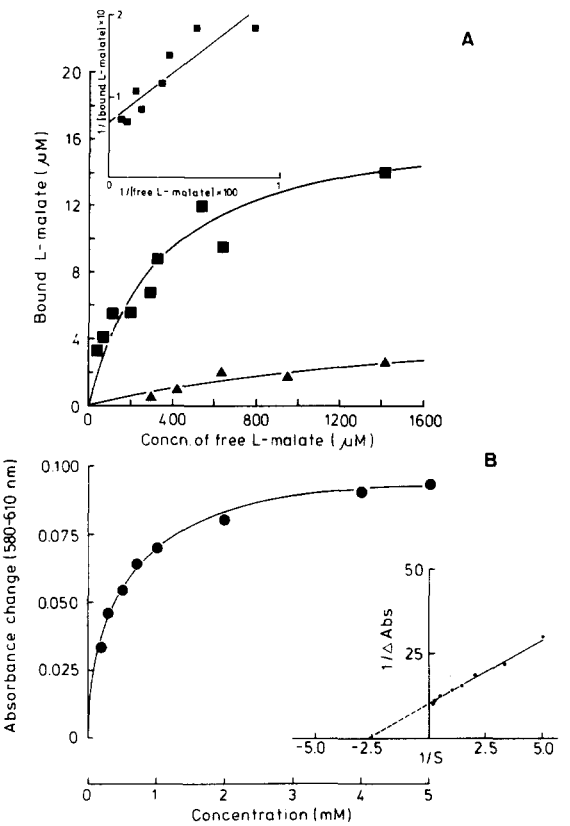


Fig. 5. (A) (■———■) represents binding of L-malate to dicarboxylic acid binding protein preparation in the absence of D-malate. Reciprocal values of these experimental data are plotted in the inset. (▲———▲) shows the effect of 0.2 mM D-malate on the binding of L-malate to dicarboxylic acid binding protein preparation. (B) Uptake of L-malate by intact luminal-membrane vesicles as studied by spectrophotometry. The inset shows a Lineweaver-Burk analysis of the data.

dicarboxylic acid binding protein. The rate of uptake of succinate as measured by spectrophotometry [14] shows a very rapid increase at low succinate concentrations (0.2–1 mM). In the range of 1–5 mM succinate the increase was less pronounced, and the optical response was almost constant at higher concentrations, suggesting that the renal transport of succinate approached saturation. The results can be described by an equation, which in form is identical to the Michaelis-Menten equation and reported in recent papers [15,32]. To calculate apparent K_m value (i.e., succinate concentration that gives half maximal uptake) for the transport of succinate the results were plotted in double-reciprocal plot (Fig. 4B, inset), and from the Lineweaver-Burk analysis of the data apparent K_m value was found to be 0.22 mM, which is apparently in good agreement with the K_d value of succinate interactions to the dicarboxylic acid binding protein preparation. Similar experiments were performed with L-malate (Figs. 5A and 5B), which is another preferred substrate of renal dicarboxylic acid transport system. In this case the values obtained for K_d and K_m were 0.33 mM and 0.37 mM, respectively. Moreover, we have also examined the effect of D-malate, which has higher affinity for the transport system than does L-malate on the interactions of L-malate with the dicarboxylic acid binding protein preparation. According to expectations the presence of D-malate (0.2 mM) strongly inhibits the binding of L-malate to dicarboxylic acid binding protein.

In conclusion the results presented in this communication indicate the successful preparation of a partially purified dicarboxylic acid binding protein from luminal-membranes with binding properties consistent with its involvement in dicarboxylic acid transport. The protein seems to be present in larger amounts than the Na^+/D-glucose transporter [6], which will facilitate further biochemical studies. However, final demonstration of transport capability by reconstitution experiments has yet to be achieved.

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