ACTIVATION OF PHOSPHORYLATING VESICLES BY NET TRANSFER OF PHOSPHATIDYL CHOLINE BY PHOSPHOLIPID TRANSFER PROTEIN

Y. Kagawa, L. W. Johnson and E. Racker

From the Section of Biochemistry & Molecular Biology
and the Graduate School of Nutrition
Cornell University, Ithaca, New York 14850

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SUMMARY: Vesicles formed with phosphatidyl ethanolamine, phosphatidyl choline, cardiolipin, coupling factors and hydrophobic proteins from bovine heart mitochondria catalyzed a rapid $^{32}P_i$-ATP exchange. When phosphatidyl choline was deleted during the assembly of the vesicles, little $^{32}P_i$-ATP exchange was observed. Exchange activity was induced by incubating such deficient vesicles with phosphatidyl choline liposomes in the presence of a phosphatidyl choline transfer protein isolated from bovine heart. Transfer of [^{32}P] phosphatidyl choline was demonstrated by isolation of the activated vesicles by sucrose density centrifugation.

A phospholipid exchange protein which catalyzes the exchange of phospholipids between subcellular organelles has been described and purified (1-5). Previous attempts to demonstrate that this protein also catalyzes a net transfer of phospholipid had only limited success (4).

It is the purpose of this communication to show that vesicles reconstituted with hydrophobic mitochondrial proteins (6), phosphatidyl ethanolamine and cardiolipin which did not catalyze $^{32}P_i$-ATP exchange (7) were converted into active particles by incubation with phosphatidyl choline and the exchange protein. A net transfer of [^{32}P] phosphatidyl choline was also demonstrated. Since net transfer has thus been established we call the catalyst a transfer protein.

MATERIALS AND METHODS

Phosphatidyl choline and phosphatidyl ethanolamine from soybean phospholipids were purified as previously described (7). Cardiolipin and egg phosphatidyl choline were purchased from General Biochemical Corporation. The solvents of these phospholipids were removed at room temperature under nitrogen, then small amounts of ether were added to the residue and the evaporation was

Abbreviations: PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; F_1, coupling factor 1 (ATPase); OSCP, oligomycin sensitivity-conferring protein.

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repeated. To prepare liposomes the residue (5 to 50 μmoles) was suspended in 1 ml of 1 mM DTT - 10 mM Tricine-KOH, pH 8.0 and sonicated in a Narda Sonifier under nitrogen at room temperature for 10 min. ^{32}P-labeled phosphatidyl choline (33,000 cpm/μmole) was isolated from rat livers and ^{14}C-triolein was purchased from Amersham/Searle and purified by thin layer chromatography (5). Other chemicals, F_1, OSCP and other preparations were obtained as previously described (6, 7). The phosphatidyl choline exchange protein was isolated from bovine heart by the procedure of Wirtz and Zilversmit (8) slightly modified to yield a preparation with a specific activity of 120 to 160 units per mg protein. One unit is defined as that amount of protein which catalyzes the exchange of 80 p moles of phosphatidyl choline from ^{32}P-labeled liposomes containing 300 nmoles of phosphatidyl choline to 12.5 mg of mitochondria in 40 minutes at 37° C.

Analysis of Net Phospholipid Transfer Reaction

Step 1: Reconstitution of phosphatidyl choline-less vesicles - The mitochondrial hydrophobic protein fraction (20 mg) obtained between 25 and 40% ammonium sulfate saturation (7) was mixed at 4° with 40 μmoles of phosphatidyl ethanolamine, 10 μmoles of cardiolipin, 80 mg of sodium cholate, 2 μmoles of dithiothreitol, 200 μmoles of ammonium sulfate, 250 μmoles of sucrose, 0.1 μmole EDTA and 20 μmoles of Tricine-KOH in a final volume of 3 ml (pH 8.0). The mixture was dialyzed at 4° against 100 volumes of 10% methanol, 1 mM dithiothreitol, 10 mM Tricine-KOH (pH 8.0), 0.2 mM EDTA for 20 hours with 2 changes of the dialysis medium.

Step 2: Incorporation of phosphatidyl choline into phosphatidyl choline-less vesicles - Vesicles containing 250 μg of hydrophobic protein were incubated for 60 min at 30° in a final volume of 0.3 ml in the presence of 5 μmoles of Tricine-KOH (pH 8.0) and the indicated amounts of transfer protein and phosphatidyl choline liposomes.

Step 3: Separation of integrated vesicles - The mixture was layered with a Pasteur pipette onto 8 ml of 0.3 M sucrose in a polycarbonate centrifuge tube and sedimented at 0° for 30 min at 165,000 x g in the Spinco 50 Ti rotor. The turbid floating layer containing the liposomes was carefully re-

moved with a Pasteur pipette. The surface of the sucrose solution was washed twice with 1 ml of water to remove residual liposomes. The sucrose solution was then aspirated and the wall of the tube wiped dry.

Step 4: Reconstitution with coupling factors - The pellet was homogenized in the centrifuge tube with a fitted teflon homogenizer with 0.1 ml of solution containing 250 mM sucrose, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM EDTA, 500 μg bovine serum albumin, 40 μg of F_1 and 10 μg OSCP. The suspension was incubated at 30° for 10 min to allow the coupling factors to combine with the vesicles.

Step 5: Assays - To the reconstituted vesicles 0.9 ml of a solution containing 10 μmoles of $^{32}P_i$ (70,000 cpm per μmole), pH 7.4, 5 μmoles of $MgCl_2$ and 5 μmoles of ATP were added and the mixture was incubated 10 min at 30°. After addition of 0.1 ml of 50% trichloroacetic acid and centrifugation, 0.5 ml of the supernatant was assayed for [^{32}P] ATP as previously described (6). The pellet was suspended in 1 ml of distilled water and extracted with 5 ml of a 1:1 mixture (v/v) isobutanol-benzene using a Vortex mixer for 1 min. The isobutanol-benzene layer was extracted twice with 3 ml of H_2O to remove residual $^{32}P_i$. After centrifugation to remove water droplets the isobutanol-benzene extract was counted without scintillator (Cerenkov-light) as described by Plesmus and Bunch (9). This measured the [^{32}P] transferred as phosphatidyl choline into the vesicles. When ^{14}C-triolein was used it was counted after the addition of scintillator (6).

RESULTS AND DISCUSSION

It can be seen from Table I that vesicles reconstituted with hydrophobic proteins and with phosphatidyl ethanolamine and cardiolipin at a molar ratio of 4:1 catalyze a low rate of $^{32}P_i$-ATP exchange even when phosphatidyl choline was added to the assay. However, when these relatively inactive vesicles were incubated with phosphatidyl choline in the presence of the phosphatidyl choline transfer protein a pronounced stimulation of $^{32}P_i$-ATP exchange was observed. The $^{32}P_i$- exchange rates in the absence of PC and transfer protein were somewhat variable and critically dependent on the ratio of phosphatidyl ethanolamine to

TABLE I

Activation of Phosphatidyl Choline-less Vesicles by Phosphatidyl Choline in the Presence of Transfer Protein

Activation of the phosphatidyl choline-less vesicles (250 μg protein) with 0.6 μmole P of phosphatidyl choline liposomes and 200 units of transfer protein were performed as described under "Methods". Complete vesicles were reconstituted as described (6) with 1 mg of the hydrophobic protein fraction and 2 μmoles of phosphatidyl choline, 2 μmoles of phosphatidyl ethanolamine and 0.5 μmole of cardiolipin.

Addition	$^{32}P_i$-ATP exchange nmoles per 10 min per mg protein
PC-less vesicles + PC	1.5
PC-less vesicles + PC + transfer protein	82.5
Vesicles reconstituted with PC + PE + cardiolipin	508.0

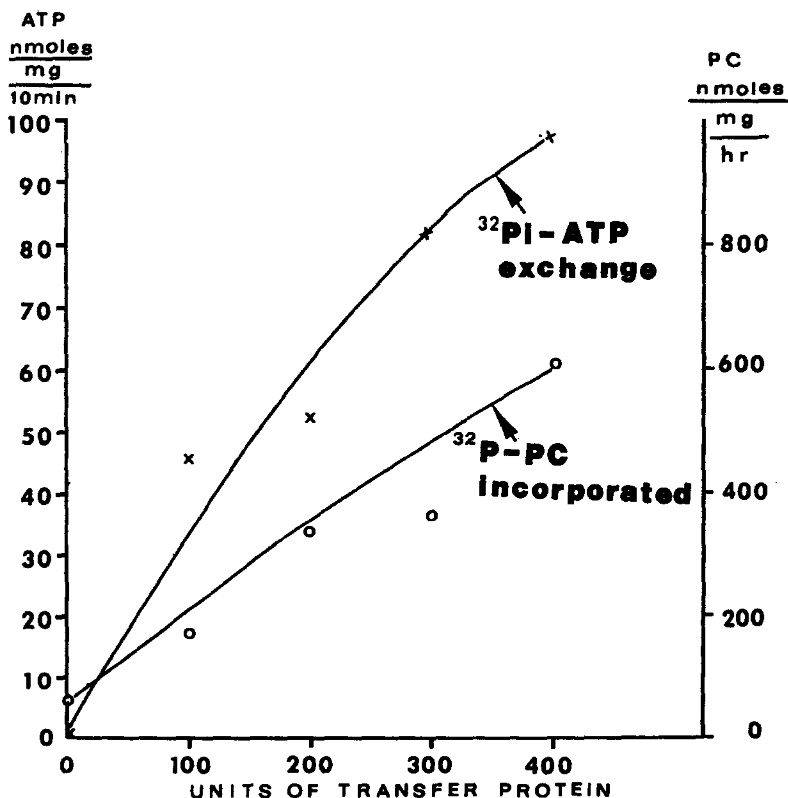

Fig. 1. Effect of the concentration of transfer protein on $^{32}P_i$-ATP exchange and net ^{32}P-PC transfer into PC-less vesicles - The experiment was performed as described under "Methods" with 250 μg protein of PC-less vesicles, 1 μmole P of PC-liposomes and the indicated amounts of transfer protein.

cardiolipin. When this ratio was increased the rate was considerably larger than that shown in Table I; when the ratio was decreased the control rate was virtually zero, but the $^{32}P_i$-ATP exchange in the presence of the transfer protein was only 25 to 30% of the rate at the 4:1 ratio. It may also be noted from Table I that even the highest rates achieved by subsequent incorporation of phosphatidyl choline into the vesicles were much lower than those obtained by the standard conditions in which all three phospholipids were incorporated initially.

As shown in Fig. 1, the rate of the $^{32}P_i$-ATP exchange was dependent on the amount of transfer protein added. To show the actual transfer of phospholipid, [^{32}P] phosphatidyl choline was used in this experiment and incorporation of radioactivity into the reconstituted vesicles was measured as described under "Methods". It can be seen from Fig. 1 that the incorporation of radioactivity into the PC-less vesicles is proportional to the amount of transfer protein added during step 2. This experiment thus demonstrates the net transfer of phosphatidyl choline into the PC-less vesicles and also establishes the validity of the procedure used to separate the reconstituted vesicles from the excess of PC-liposomes that have not been incorporated.

The effect of varying the concentration of phosphatidyl choline during the second step of reconstitution is shown in Fig. 2. The $^{32}P_i$-ATP exchange rate reached an optimal value when PC-liposomes equivalent to 1 μmole phosphorus were used; higher concentrations yielded slightly lower rates. In contrast, the incorporation of [^{32}P] phosphatidyl choline into the vesicles was linearly related to the amounts of the phospholipid added. This difference in response of the two measured activities is not unexpected in view of the previous observation (7) that the exchange rate varies with the input ratio of the different phospholipid species. The experiments were also carried out with only one radioactive isotope ($^{32}P_i$ or [^{32}P] PC) present. The results were the same, ruling out possible transfer of labeled phosphate between ATP and phospholipid.

Egg phosphatidyl choline could replace soybean phosphatidyl choline. How-

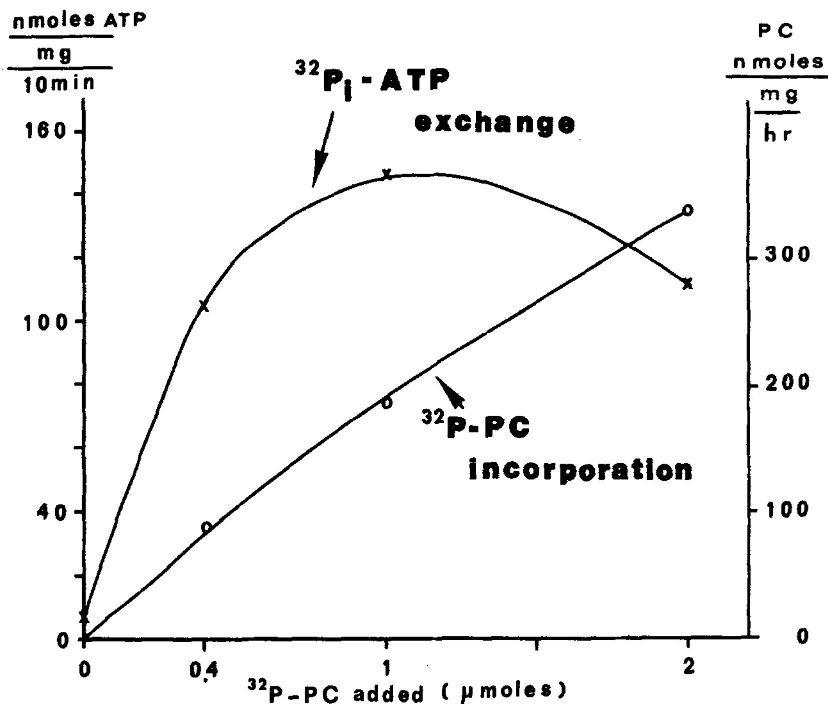

Fig. 2. Effect of phosphatidyl choline concentration on formation of active vesicles - The experiment was performed as described in the legend of Fig. 1 with 200 units of transfer protein and the indicated amounts of PC.

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ever, when the procedure was reversed and phosphatidyl choline plus cardiolipin was used in the first step and phosphatidyl ethanolamine in the second, the $^{32}P_i$-ATP exchange was negligibly low after incubation with or without transfer protein. There was also no incorporation of $[^{32}P]$ phosphatidyl ethanolamine into the vesicles. Sticking of PC-liposomes to PC-less vesicles was also measured by incubating PC-liposomes containing ^{14}C-triolein with the PC-less vesicles. Less than 20 nmoles per mg per hour of phospholipid liposomes were recovered in the isolated reconstituted vesicles irrespective of whether transfer protein was present or absent.

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