THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 262, No. 33, Issue of November 25, pp. 15979–15983, 1987
Printed in U.S.A.

Reconstitution of the Malate/Aspartate Shuttle from Mitochondria*

(Received for publication, April 22, 1987)

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The isolated aspartate/glutamate carrier and oxoglutarate carrier from mitochondria were coreconstituted into phospholipid vesicles. Reconstitution of the functionally active carrier proteins with high protein/lipid ratios was achieved by detergent removal on hydrophobic ion-exchange columns. A simplified version of the mitochondrial malate/aspartate shuttle was constructed by inclusion of glutamate-oxaloacetate transaminase and the substrates aspartate and oxaloacetate within the interior of the liposomes. Addition of external glutamate led to internal production of oxoglutarate which could be exchanged against externally added labeled malate. The reconstitution procedure was characterized with respect to the optimum ratio of reconstituted carrier proteins, the lipid concentration, and the concentration of internal substrates.

It is generally accepted that the malate/aspartate shuttle plays an important role in the transfer of reducing equivalents from the cytosol to the mitochondria in most tissues (1). This shuttle, first proposed by Borst (2, 3), has been demonstrated to operate in intact cells of various tissues (1, 4–10) and in intact isolated mitochondria when supplemented with the corresponding external enzymes and substrates (11–16). The basic idea of the malate/aspartate shuttle (2, 3) was developed considering the presence of malate dehydrogenase and aspartate aminotransferase in high activity both in the interior of the mitochondria and in the cytosol. Only after the various transport functions of the inner mitochondrial membrane had been extensively investigated (see Ref. 17 for review), were the aspartate/glutamate carrier and the oxoglutarate carrier identified as the transport systems involved in the malate/aspartate shuttle. The oxoglutarate carrier on the one hand prefers 2-oxoglutarate and malate as substrates and catalyzes an electroneutral malate^{2-}/oxoglutarate^{2-} exchange. The aspartate/glutamate carrier, on the other hand, catalyzes an electrogenic transport of (glutamate$^-$ + H$^+$) against aspartate$^-$. This electrogenic character is the reason why the complete malate/aspartate shuttle is unidirectional in energized mitochondria.

Recently the oxoglutarate carrier (18) and the aspartate/glutamate carrier (19) have been purified to homogeneity and functionally reconstituted into liposomes. Several substrate carriers from the inner mitochondrial membrane or from other membranes have so far been reconstituted separately. This is an essential step for the identification of a transport protein and for the elucidation of its function and mechanism. However, in the case of metabolic shuttle mechanisms, usually two or even more carriers cooperate within the natural membrane to accomplish specific functions. One of the best examples for such a functional cooperation is the malate/aspartate shuttle, which includes the two carriers for aspartate/glutamate exchange and for oxoglutarate/malate exchange, respectively.

In this paper we report the functional coreconstitution of the oxoglutarate and the aspartate/glutamate carrier into liposomes. The experimental data show that this system represents a basis for further studies on the function and the regulation of the reconstituted malate/aspartate shuttle.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyapatite (Bio-Gel HTP) and Dowex AG 1-X8 were purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Serva, L-[U-^{14}C]malate from Amersham Buchler, phospholipids (phosphatidylcholine from fresh turkey egg yolk) and Pipes[1] from Sigma, glutamate oxaloacetate transaminase and pyridoxalphosphoric acid from Boehringer Mannheim. Triton X-114 and $C_{13}E_{10}$ (tridecyl decaoxyethylene ether) were obtained from Sigma, $C_{12}E_8$ (dodecyl octaoxyethylene ether) from Kouyouh Trading Company, Tokyo, and the maxifluor scintillation mixture from J. T. Baker Chemical Co. All other reagents were of the highest purity commercially available.

Isolation of the Oxoglutarate Carrier—Bovine heart mitochondria were prepared as described in Ref. 20. The oxoglutarate carrier was purified from these mitochondria by the method introduced by Bisaccia *et al.* (18) including some modifications described recently (21). The second and the third fractions of the eluate obtained after hydroxyapatite/celite chromatography of the mitochondrial Triton X-114 extract, which contain purified oxoglutarate carrier protein, were combined and used for the reconstitution.

Isolation of the Aspartate/Glutamate Carrier—The aspartate/glutamate carrier was purified from bovine heart mitochondria as described by Krämer *et al.* (19). In most experiments partially purified carrier after the second step of purification (Sephadex G-25 column) was used, which does not contain any glutamate- or aspartate-metabolizing proteins but still contains considerable amounts of adenine nucleotide carrier. The important experiments, however, which prove the shuttle activity (*e.g.* that of Table I) have all been carried out also with the purified protein after hydroxyapatite-HPLC (19), leading to identical results.

Incorporation of the Oxoglutarate and the Aspartate/Glutamate Carrier into Liposomes—Liposomes were prepared as described previously (22) by sonication of 100 mg/ml egg yolk phospholipids in water for 60 min. Coreconstitution of the aspartate/glutamate and the oxoglutarate carrier into liposomes was performed by detergent removal on a hydrophobic ion-exchange column (23, 24). In this procedure the mixed micelles containing detergents, proteins, and phospholipids are repeatedly passed through columns filled with

* This investigation was supported in part by a grant from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviation used is: Pipes, 1,4-piperazinediethanesulfonic acid.

amberlite in order to remove the detergents and to allow the formation of proteoliposomes.

The standard composition for reconstitution was: 225 μl of solubilized oxoglutarate carrier (10–16 μg of protein of purified carrier in 2.5% Triton X-114), 400 μl of solubilized aspartate/glutamate carrier (5–10 μg of protein in the case of purified carrier, 150–200 μg of protein in the case of partially purified carrier, both in 0.4% $C_{12}E_8$), 4.7 mg of phospholipids in the form of sonicated liposomes, 0.18 mg of glutamate-oxaloacetate transaminase, 15 mM oxaloacetate, 1.6 mM aspartate, and 10 mM Pipes, pH 6.5, in a final volume of 0.8 ml. The high internal concentration of oxaloacetate was necessary because of the lack of malate dehydrogenase activity in the reconstituted system. Under these conditions oxaloacetate cannot be regenerated from malate taken up by the oxoglutarate carrier. Deviations from this composition are indicated in the legends of the corresponding tables and figures. After vortexing, this mixture was passed 15 times through an amberlite column (0.5-cm diameter) pre-equilibrated with buffer containing Pipes, oxaloacetate, and aspartate at the same concentrations as described for the reconstitution mixture. In each experiment the amount of amberlite to be used was adjusted to an amberlite/detergent ratio of about 50 (w/w). After this step, large proteoliposomes were formed in a solution containing oxaloacetate (15 mM), aspartate (1.6 mM) and glutamate-oxaloacetate transaminase (0.16 mg/ml). The two carrier proteins were incorporated into the proteoliposomes most likely in the same proportion in which they were added to the reconstitution mixture. All the operations were performed at 0 °C, except for the removal of the detergents by amberlite which was carried out at room temperature.

Transport Measurements—The external substrates (aspartate and oxaloacetate) were removed by passing the proteoliposomes (500 μl of the amberlite eluate) through a Sephadex G-75 column (0.7 × 15 cm) pre-equilibrated with 50 mM NaCl and 10 mM Pipes, pH 6.5 (Sephadex-buffer). The first 750 μl of the slightly turbid eluate from the Sephadex column, containing the proteoliposomes, were collected, distributed in reaction vessels (150 μl each) and kept at 25 °C for 4 min. To these samples 10 mM glutamate in 10 μl of Sephadex buffer or 10 μl of Sephadex buffer alone was added. After 5 min of preincubation, the proteoliposomes were used for transport measurements by the inhibitor-stop method (25). Transport was initiated by adding 0.1 mM [^{14}C]malate (30,000–40,000 dpm). After 5 min (unless otherwise indicated), the reaction was stopped by the addition of 20 mM pyridoxalphosphate, which is a rapid and efficient stopping inhibitor of both the aspartate/glutamate carrier (26) and the oxoglutarate carrier.[2] In control samples pyridoxalphosphate was added together with the labeled substrate at time zero. In order to remove the external radioactivity, each sample was applied onto a column (0.5 × 5 cm) of Dowex AG 1-X8, 50–100 mesh, acetate form (26). The liposomes eluted by the addition of 1 ml of 40 mM sodium acetate were collected in scintillation vials, mixed with 4 ml of scintillation mixture, and counted.

RESULTS

Reconstitution of Shuttle Activity—For functional reconstitution of the malate/aspartate shuttle both the oxoglutarate and the aspartate/glutamate carrier must be present in one single liposome. Therefore, it is essential to achieve a high protein/lipid ratio in the reconstituted proteoliposomes. This important prerequisite cannot be fulfilled when using the common freeze-thaw-sonication procedure (27, 28) for reconstitution, which is usually characterized by a protein/lipid ratio (w/w) of less than 10^{-3}. Unfortunately, also the dialysis method for reconstitution (29), which generally leads to high protein/lipid ratios, cannot be applied in this case. The detergents used for solubilization and purification of these two carrier proteins are not suited for dialysis and both the oxoglutarate and the aspartate/glutamate carrier do not endure dialyzable detergents, *e.g.* bile acids or octyl glucoside.

For these reasons we have applied a method recently developed for the functional reconstitution of membrane proteins (24), which is based on the removal of detergents by chromatography on hydrophobic ion exchange resins (23). This method leads to the incorporation of sufficiently large amounts of protein into the vesicular membranes. In general 20–40% of the proteoliposomes formed by this procedure carried both carrier proteins.

For directly measuring the activity of the reconstituted malate/aspartate shuttle, this complex redox shuttle had to be simplified to some extent. Fig. 1 illustrates the general experimental design used to measure the combined activity of the aspartate/glutamate carrier, the oxoglutarate carrier, and the glutamate-oxaloacetate transaminase. Liposomes which contain oxaloacetate, aspartate, and glutamate-oxaloacetate transaminase in the internal space, and the two reconstituted carriers within the membrane are preincubated with externally added glutamate. This leads to the formation of 2-oxoglutarate inside the liposomes by the action of the aspartate/glutamate carrier and glutamate-oxaloacetate transaminase. Aspartate is exported in exchange for the glutamate taken up and is regenerated from oxaloacetate by the activity of glutamate-oxaloacetate transaminase. This leads to further uptake of glutamate and additional formation of oxoglutarate inside. Upon adding [^{14}C]malate, the labeled substrate can now be taken up in exchange against the accumulated internal oxoglutarate by the action of the oxoglutarate carrier. The concerted activities cannot take place when glutamate is omitted in the preincubation. Thus, the difference in the amount of malate taken up in the presence and in the absence of glutamate represents the activity of the reconstituted shuttle. Thus, the differences between the simplified shuttle and the *in vivo* system are: 1) lack of the dehydrogenase reactions and 2) lack of external transaminase reaction in the vesicle system. However, the central part, *i.e.* the carrier functions, can be well studied in this reconstituted system of the malate/asparate shuttle.

The time course of [^{14}C]malate uptake in reconstituted liposomes is shown in Fig. 2. Obviously, also when the proteoliposomes were preincubated in the absence of glutamate, there is a considerable uptake of labeled malate. This can be easily explained since the oxoglutarate carrier catalyzes also

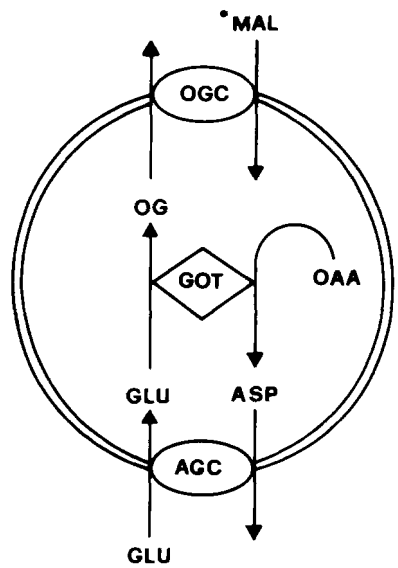

FIG. 1. **Scheme of the reconstituted malate/aspartate shuttle.** The proteoliposomes contain the carriers for oxoglutarate (*OGC*) and aspartate/glutamate (AGC) and, in the internal volume, the glutamate-oxaloacetate transaminase (*GOT*) and the substrates oxaloacetate (*OAA*) and aspartate (*ASP*). Glutamate (*GLU*) and oxoglutarate (*OG*) are the substrates produced inside the liposomes upon addition of external glutamate. [^{14}C]Malate (**MAL*) is taken up by the oxoglutarate carrier in exchange for the accumulated oxoglutarate.

[2] C. Indiveri, R. Krämer, and F. Palmieri, unpublished results.

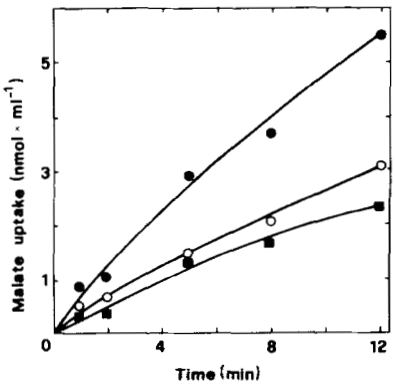

FIG. 2. **Time course of [^{14}C]malate uptake in reconstituted liposomes.** Proteoliposomes prepared as described under "Experimental Procedure" were preincubated in the presence (●) or absence (○) of 10 mM glutamate. 0.1 mM [^{14}C]malate was added at time zero, and the uptake was stopped by 20 mM pyridoxalphosphate after the indicated time interval. The values used for curve (■) represent the difference between malate uptake in liposomes preincubated with and without glutamate.

a slow exchange of malate against oxaloacetate which is present at 15 mM concentration inside the liposomes. The amount of malate taken up by the vesicles preincubated in the absence of glutamate is about 50% of the value found in proteoliposomes preincubated with glutamate. This means that in the liposomes containing both carriers (about 30% of the total vesicles) the relative contribution of the glutamate-induced uptake of labeled malate (against oxoglutarate) is much higher, since the basic activity (oxaloacetate exchange against malate) occurs in all the liposomes which contain the oxoglutarate carrier, whereas the glutamate-induced function of the malate/aspartate shuttle only occurs in liposomes which contain both the oxoglutarate and the aspartate/glutamate carrier.

Especially when considering the basic rate of oxaloacetate/malate exchange, one has to be very serious about controls for the observed activity of the aspartate/malate shuttle. For this purpose we omitted one component of the test system (Fig. 1) after the other in control experiments. This is summarized in Table I. It is obvious, that the lack of any of the important compounds, *i.e.* glutamate-oxaloacetate transaminase (experiment 2), oxaloacetate (experiment 3), or aspartate (experiment 4), completely abolishes the activity of the reconstituted aspartate/malate shuttle. The stimulation of malate uptake by glutamate is also eliminated when aminooxyacetate, an inhibitor of the glutamate-oxaloacetate transaminase (30), was added to the complete reaction mixture (experiment 5). All these results clearly indicate the absolute requirement of glutamate-oxaloacetate transaminase, oxaloacetate, and aspartate inside the liposomes for the activity of the reconstituted shuttle, *i.e.* for the glutamate-induced increase of malate uptake.

So far, the importance of substrates and glutamate-oxaloacetate transaminase for the function of the malate/aspartate shuttle have been tested (Table I). The final experiment is even more conclusive, since it demonstrates the requirement of the two carriers reconstituted in the same liposome. Instead of the simple control experiment which would consist in omitting one or both carriers in the reconstituted system, in this experiment (experiment 6 of Table I), all the conditions were exactly the same as in the basic reconstitution experiment (experiment 1), except that the two carriers have first been reconstituted separately in different pools of liposomes of the same composition. After reconstitution the two pools have been mixed. The complete lack of the shuttle activity proves the importance of functional coreconstitution of the oxoglutarate and the aspartate/glutamate carrier.

It may be noted that, when aspartate was omitted from the system (experiment 4 of Table I), there is a considerable uptake of [^{14}C]malate already in the absence of glutamate. The observed uptake is in fact more than two times higher than that of the basic experiment (experiment 1). This result can be explained by the fact that aspartate when present internally, causes a strong inhibition of the basic malate/oxaloacetate exchange. This explanation is supported by the finding that aspartate inhibits the [^{14}C]oxoglutarate/oxoglutarate exchange in liposomes reconstituted with purified oxoglutarate carrier[2] and is in agreement with previous observations by Sluse *et al.* (31) in intact mitochondria.

Parameters of Reconstitution—In order to optimize and to characterize the reconstitution system, the influence of several parameters on the activity of the reconstituted shuttle was investigated.

Table II shows the dependence of the shuttle activity on the relative amounts of the two carriers used for reconstitution. It can be seen that the activity of the malate/aspartate shuttle reaches a maximum value when well-defined ratios of aspartate/glutamate and oxoglutarate carrier were used. As might be expected, a surplus of either carrier causes a pronounced decrease in the activity. This decrease, however,

TABLE I

Functional reconstitution of the malate/aspartate shuttle: effect of removing basic components from the reconstitution mixture and importance of coreconstitution of the two carriers

In experiment 1 the conditions were the same as described under "Experimental Procedures." Abbreviations: AGC, aspartate/glutamate carrier; OGC, oxoglutarate carrier; GOT, glutamate-oxaloacetate transaminase.

Experiment	[^{14}C]Malate transported		
	After preincubation		Difference between activity with and without glutamate preincubation
	Without glutamate	With glutamate	
	nmol/ml × 5 min		
1 Coreconstituted AGC and OGC, internal GOT, Oxaloacetate Aspartate	3.6	6.8	3.2
2 Same as experiment 1 without GOT	2.9	2.7	−0.2
3 Same as experiment 1 without oxaloacetate	1.4	1.2	−0.2
4 Same as experiment 1 without aspartate	8.7	8.7	0
5 Same as experiment 1 plus 10 mM aminooxyacetate	2.0	2.1	0.1
6 Same as experiment 1, but reconstitution of the two carriers separately in different liposomes	1.5	1.6	0.1

TABLE II

Dependence of the activity of the reconstituted malate/aspartate shuttle on the relative amount of carrier proteins

Conditions as described under "Experimental Procedures," except that different amounts of aspartate/glutamate carrier and of oxoglutarate carrier were used. The reported detergent/lipid ratio was calculated on the basis of the two detergents present in the aspartate/glutamate carrier and the oxoglutarate carrier preparations.

Reconstituted carriers		Detergent/lipid	[^{14}C]Malate transported (difference between activity with and without glutamate preincubation)
Asp/Glu	Oxoglutarate		
μl of carrier preparation		*w/w*	*nmol/ml × 5 min*
540	90	0.9	0.1
400	210	1.5	3.9
300	300	1.9	3.9
210	400	2.6	0.8
90	540	3.0	0.2

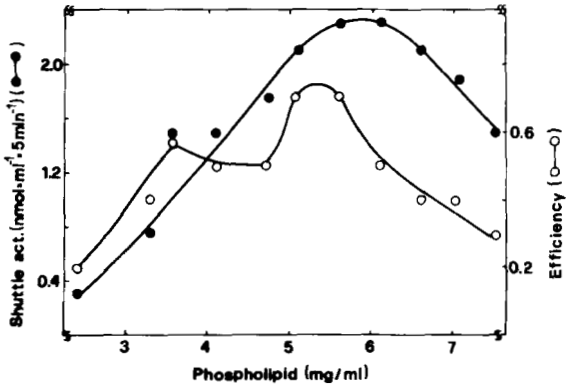

FIG. 3. **Dependence of the reconstituted malate/aspartate shuttle on the phospholipid concentration.** The proteoliposomes were prepared as described under "Experimental Procedures" except that phospholipids were used at the indicated concentrations and internal oxaloacetate was 9 mM. The efficiency of the shuttle (see text) is expressed as the ratio between malate uptake in liposomes preincubated with and without glutamate.

could also be due to an unfavorable detergent/lipid ratio during preparation of the mixed micelles. In the samples where the highest activity is found, the detergent/lipid ratio varies between 1.5 and 1.9. Therefore, the decrease in the shuttle activity observed in the presence of 210 μl of aspartate/glutamate carrier and 400 μl of oxoglutarate carrier (line 4) with respect to the opposite situation (line 2) might be accounted for by an increase in the detergent/lipid ratio. Another explanation, however, could be that addition of higher amounts of Triton X-114 (together with the oxoglutarate carrier) in the experiment of line 4 causes inactivation of the aspartate/glutamate carrier during formation of the mixed micelles. It has to be pointed out, that in all cases the detergents have been removed at the end of the reconstitution procedure.

Since the amount of phospholipids seems to be an important criterion, experiments were carried out in order to determine the dependence of the shuttle activity on the concentration of phospholipids (Fig. 3). The specific activity of the shuttle increases on increasing the amount of phospholipids in the reconstitution system, reaching a maximum in the presence of 5.5–6.0 mg of phospholipids/ml. At higher lipid concentrations the activity decreases. In Fig. 3 an additional parameter, the efficiency of the shuttle, is given. This parameter means the ratio (not the difference!) between the activities in the presence and in the absence of glutamate. It can be seen that the efficiency shows a dependence on added phospholipids more or less similar to that observed in the case of the shuttle activity. The highest efficiency, however, is reached at a concentration somewhat lower than the corresponding maximum value of the shuttle activity.

In further experiments the concentration of the internal substrates which are necessary for the entry and the transamination of glutamate was varied. The activity of the reconstituted malate/aspartate shuttle is strongly dependent on the intraliposomal concentration of aspartate (Fig. 4*A*) showing a classical Michaelis-Menten behavior with a half-maximal activity at approximately 0.3 mM aspartate. This observation is in perfect agreement with a catalytic role of aspartate in the reconstituted shuttle (*cf.* Fig. 1). On the other hand, oxaloacetate has to be present in much higher concentrations in the interior of the liposomes for high rates of reconstituted shuttle activity (Fig. 4*B*). This can be envisaged since in the experimental conditions (Fig. 1) of the reconstituted shuttle, oxaloacetate is a noncatalytic substrate, which is consumed during the function of the reconstituted system.

DISCUSSION

The successful reconstitution of the malate/aspartate shuttle is demonstrated by the results shown in Table I. There is clear evidence that we have in fact measured the activity of the combined system as described in Fig. 1, since the crucial point, *i.e.* the glutamate-stimulated uptake of malate is definitely dependent on the presence of the appropriate substrates and the enzyme glutamate-oxaloacetate transaminase inside the vesicles and on the simultaneous presence of the two carrier systems in the liposomal membranes.

The critical step in the reconstitution procedure is the coreconstitution, *i.e.* the incorporation of the aspartate/glutamate carrier and the oxoglutarate carrier into the same liposomes. Several difficulties had to be overcome for the successful coreconstitution. (i) The two carriers are solubilized in different detergents. (ii) The aspartate/glutamate carrier becomes inactivated by long exposure to or high amounts of Triton X-114. (iii) The protein/lipid ratio of the reconstituted liposomes must be reasonably high in order to make probable the simultaneous presence of functionally active molecules of each of the two carrier proteins in one single liposome. (iv) The proteoliposomes must be large enough (at least 100 nm in diameter) because the amount of substrates and glutamate-oxaloacetate transaminase in the inside of the proteoliposomes should not be limiting.

Only by the use of the recently developed reconstitution

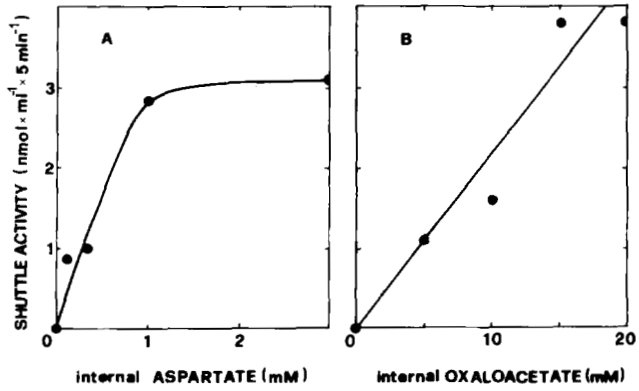

FIG. 4. **Dependence of the reconstituted malate/aspartate shuttle on the concentration of internal aspartate and oxaloacetate.** The proteoliposomes were prepared as described under "Experimental Procedures" except that the concentration of aspartate (*A*) or oxaloacetate (*B*) was varied as indicated.

method based on hydrophobic chromatography was it possible to meet all the above-mentioned prerequisites. There is, however, still the difficulty of creating different kinds of liposomes; (*a*) proteoliposomes containing only active oxoglutarate carrier, (*b*) vesicles containing only active aspartate/glutamate carrier, and (*c*) the coreconstituted proteoliposomes containing both carriers in a functionally active state. The amount of the population of interest, *i.e.*, the third fraction, reaches about 40% of total liposomes in optimum preparations (experiments not shown).

For functional reconstitution of the aspartate/malate shuttle, several parameters turned out to be important. First, it should be mentioned that the substrates, which have to be present inside the liposomes (*cf.* Fig. 1), had to be removed from the external volume for three reasons. (i) Oxaloacetate would inhibit the entry of malate, (ii) aspartate would inhibit the entry of glutamate, and (iii) oxaloacetate together with glutamate would function as substrates for external glutamate-oxaloacetate transaminase, since the enzyme is not completely removed from the outside by passage over Sephadex G-75. Furthermore, the internal concentration of the catalytic substrate aspartate had to be low, since it inhibits the oxoglutarate carrier, and the internal concentration of oxaloacetate had to be high, since in this experimental arrangement it is consumed during the function of the shuttle. Also, the ratio of the two carrier proteins during reconstitution proved to be an important factor. It should be noted that any change in the ratio of the two carriers implies not only a change in the combination of the two detergents but also a change in the detergent/lipid ratio, since in the preparation of the oxoglutarate carrier 2.5% Triton X-114 is present, whereas 0.4% $C_{12}E_8$ is present in the aspartate/glutamate carrier preparation. At the present stage it is not possible to make quantitative statements on the optimum molecular ratio of the two carriers reconstituted into the phospholipid vesicles, since the efficiency of reconstitution, *i.e.* the percentage of active carriers in the reconstituted system, is not known both for the aspartate/glutamate and for the oxoglutarate carrier.

The dependence of the shuttle activity on the phospholipid concentration as shown in Fig. 3 can be directly explained by considering the reconstitution mechanism. Below the optimum concentration of 5.5–6.0 mg phospholipid/ml not all the carrier molecules are incorporated on the one hand, and on the other hand, the liposomes formed become substantially smaller at very high protein/lipid ratios (24). At higher lipid concentrations, the number of liposomes is too high for allowing incorporation of sufficient amounts of the two different proteins into the same liposome. The efficiency, which reflects the ratio of coreconstituted liposomes (population c, see above) and total vesicles, reaches its maximum at lipid concentrations lower than those necessary for maximal activity. This means that in the range between 5.5 and 6.0 mg of phospholipid/ml, where the activity stays constant and the efficiency falls off, the number of coreconstituted vesicles remains more or less constant while the total number of liposomes increases.

In order to study the malate/aspartate shuttle of the inner mitochondrial membrane without the presence of any interfering enzyme or carrier activity, we have constructed a reconstituted system which comprises a simplified version of this important metabolic shuttle system. Mainly, the membrane part and the internal functions were used in the system described in Fig. 1. The activities of the two carriers for aspartate/glutamate and for oxoglutarate are connected by means of the appropriate substrates and the enzyme glutamate-oxaloacetate transaminase. Although the shuttle system is not complete, since the redox enzymes are still missing, the experimental conditions allow the investigation of the combined action of the two carrier proteins involved. This should be a solid basis for further studies on the interdependence and the regulation of this shuttle system.

Acknowledgment—We appreciate the support of Professor Dr. M. Klingenberg.

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