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The reconstituted carnitine carrier from rat liver mitochondria: evidence for a transport mechanism different from that of the other mitochondrial translocators

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The transport mechanism of the reconstituted carnitine carrier purified from rat liver mitochondria was investigated kinetically. The half-saturation constant (K_m) for carnitine on the internal side of the liposomal membrane (8.7 mM) was found to be much higher than that determined for the external surface (0.45 mM). The exclusive presence of a single transport affinity for carnitine on each side of the membrane indicated a unidirectional insertion of the carnitine carrier into the proteoliposomes, most probably right-side-out with respect to mitochondria. Under these defined conditions bisubstrate initial velocity studies of homologous (carnitine/carnitine) and heterologous (carnitine/acylcarnitine) antiport were performed by varying both the internal and external substrate concentrations. The kinetic patterns obtained showed that the ratio K_m/V_{max} is not influenced by the second (non-varied) substrate, which indicates a ping-pong mechanism. The carnitine carrier thus differs from all other mitochondrial carriers analyzed so far in the reconstituted state, for which a common sequential type of reaction mechanism has been found.

Introduction

The inner mitochondrial membrane contains a specific carrier system for the transport of carnitine and acylcarnitines, known as the carnitine carrier (for review, see Ref. 1). This system plays a central role in the translocation of fatty acids via acylcarnitines into the mitochondrial matrix, where the acyl groups are released to be used for fatty acid oxidation. The properties of the carnitine carrier have been extensively investigated in intact mitochondria [2–10]. This carrier catalyzes a 1:1 exchange between acylcarnitines and carnitine, which is inhibited by SH reagents like mersalyl and *N*-ethylmaleimide and by substrate analogues like sulphobetaines. Furthermore, besides the exchange reaction a unidirectional transport of carnitine is catalyzed with lower activity. Recently, we have identified and purified the carnitine carrier protein from rat liver mitochondria [11]. When functionally reconstituted, the purified protein with an apparent molecular mass of 32.5 kDa mediated the exchange reaction between carnitine and acylcarnitines, as well as the uniport mode of transport [12,13]. The substrate specificity and inhibitor sensitivity were similar to those found in intact mitochondria [11–13]. In addition, in the reconstituted system we were able to discriminate two classes of SH-groups that are functionally important for the transport activity of the carnitine carrier protein [14].

Mainly based on studies in reconstituted systems it has been proposed that the mitochondrial carriers, besides forming a structural family [15–17], also constitute a homogenous functional group of proteins, characterized by a common sequential type of reaction mechanism [1]. For a detailed functional characterization of the reconstituted carnitine carrier, and for comparison with other members of the mitochondrial carrier family, it is important to establish the orientation of the inserted protein. Moreover, investigation of the transport mechanism is of particular interest in the case of the carnitine carrier since it is the only known mitochondrial transport system which is able to catalyze both electroneutral exchange and uniport in a non H^+-compensated manner. In this paper, by applying the two-substrate kinetic analysis, it was possible to determine whether one (ping-pong mechanism) or two binding sites (sequential mechanism) have to be occu-

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Abbreviations: DTE, dithioerythritol; Pipes, 1,4-piperazinediethanesulphonic acid.

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pied before the transport reaction occurs. We showed that the exchange reaction catalyzed by the carnitine carrier proceeds via a ping-pong mechanism. Furthermore, evidence is provided that the reconstituted carnitine carrier is oriented unidirectionally in the liposomal membrane, right-side out compared to mitochondria.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, Sephadex G-50 and G-75 from Pharmacia, L-[^{3}H]carnitine from Amersham, egg-yolk phospholipids (L-α-phosphatidylcoline from fresh turkey egg yolk), cardiolipin, Pipes, Triton X-100, and L-carnitine from Sigma, L-acetylcarnitine from Serva. L-Propionylcarnitine was a gift from Prof. N. Siliprandi and Dr. F. Di Lisa. All other reagents were of analytical grade.

Purification of the carnitine carrier. The carnitine carrier was purified from rat liver mitochondria as described in Ref. 11. The completely purified protein, i.e., the third celite fraction, was used in all the experiments.

Reconstitution of the purified carnitine carrier into liposomes. The purified protein was reconstituted by removing the detergent with a hydrophobic ion-exchange column [18,19]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-2 column. The composition of the initial mixture used for reconstitution was: 380 μl of the purified carnitine carrier (1–2 μg protein in 3% Triton X-100), 100 μl of 10% egg-yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. 20, L-carnitine at the indicated concentrations, 10 mM Pipes (pH 7) in a final volume of 680 μl. After vortexing, this mixture was passed 16 times through the same Amberlite column (0.5 × 3.6 cm) preequilibrated with a buffer containing 10 mM Pipes (pH 7) and the same concentration of L-carnitine present in the starting mixture. All the operations were performed at 4°C except the passages through Amberlite, which were carried out at room temperature. Where indicated, in order to scramble the orientation of the liposomal membrane, the proteoliposomes were frozen (at −80°C) and thawed three times and then pulse-sonicated for 20 s.

Transport measurements. In order to remove the external substrate, 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 10 mM Pipes (pH 7) and 10 mM NaCl. This chromatography was performed in 3 min and at 2°C to minimize the efflux of carnitine from the proteoliposomes. The transport activity was determined by measuring the flux of labelled carnitine from outside to inside (forward exchange) or from inside to outside (backward exchange).

For backward-exchange measurements, the proteoliposomes containing internal carnitine at various concentrations (indicated as y) were prelabelled by carrier-mediated exchange equilibration. This was achieved by incubating the proteoliposomes with 0.1 mM [^{3}H]carnitine at high specific radioactivity (3 μCi/nmol) for 20 min at 25°C. After this incubation, 0.4 μM mersalyl (i.e., a concentration of SH reagent sufficient to block completely and reversibly carnitine transport) was added, and subsequently the external radioactivity was removed by passing the liposomes through a Sephadex G-75 column as described above. The concentration of carnitine in the active liposomes, i.e., the carrier-loaded liposomes, at the end of the prelabelling procedure was estimated as described in Ref. 13 with the following modifications. Proteoliposomes containing various concentrations of internal carnitine (y mM), incubated with 0.1 mM unlabelled carnitine for 20 min (to reproduce the prelabelling conditions) or with y mM unlabelled carnitine (controls), were supplemented with 0.4 μM mersalyl and passed through Sephadex G-75. Then, both samples were incubated for 60 min at 25°C in the presence of 0.1 mM external [^{3}H]carnitine plus 0.2 mM DTE (to restore the carrier activity previously inhibited by 0.4 μM mersalyl), and after this incubation period the radioactivity taken up was measured. Under these conditions the uptake of [^{3}H]carnitine is proportional to the amount of substrate present in the active liposomes. Since one can assume that in those samples incubated with y mM unlabelled external carnitine the internal concentration remains unchanged, we can estimate the concentration of carnitine present in the active liposomes of the samples incubated with 0.1 mM carnitine as $(dpm_2/dpm_1) \times y$ mM, where dpm_2 and dpm_1 represent the label taken up by the proteoliposomes incubated with 0.1 mM and y mM carnitine, respectively. With this method we found, for example, that with an initial concentration of 15 mM carnitine inside the liposomes the internal concentration of carnitine at the end of the prelabelling procedure had decreased by 15%.

Transport was started, in the case of the forward exchange, by adding labelled carnitine to the proteoliposomes or, in the case of the backward exchange, by adding unlabelled carnitine plus 0.2 mM DTE (to restore the carrier activity previously inhibited by 0.4 μM mersalyl) to the prelabelled proteoliposomes. In both cases transport was stopped by adding 0.9 mM *N*-ethylmaleimide. In control samples the inhibitor was added at time zero according to the inhibitor stop method [21]. The assay temperature was 25°C. Finally, each sample of proteoliposomes (100 μl) was passed

through a Sephadex G-50 column (0.6 × 8 cm) in order to separate the external from the internal radioactivity. The liposomes eluted with 1.3 ml of 50 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted.

The isotope equilibration kinetics were followed by stopping the transport reaction after different time intervals. In the case of the forward exchange, the experimental values were corrected by subtracting the respective control. The corrected data were then fitted by a computer program according to a first-order process equation, allowing the initial transport rate to be calculated, expressed in μmol/min per mg protein. In the case of the backward exchange, the decrease in radioactivity inside the liposomes (until equilibrium was reached) was fitted according to a single exponential decay equation, from which the first-order rate constant k was derived. The backward-exchange rates were expressed as apparent velocities v' (mM/min), i.e., the product of k and the substrate concentration inside the liposomes measured as described above. The values of v' are directly proportional to the actual transport rate (see Ref. 22). Therefore, the relative backward-exchange velocities were suitable for all kinetic investigations carried out in the present paper. It should be emphasized that the backward-exchange method was absolutely essential for measuring carnitine carrier kinetics under particular conditions, i.e., in experiments where low internal concentrations are combined with high external concentrations that are kinetically not limiting for the exchange measurement. In this case the isotope equilibrium that is reached, i.e., $dpm_{in}/dpm_{ex} = V_{in}[S]_{in}/V_{ex}[S]_{ex}$ (V, volume; [S], substrate concentration), is drastically in favour of the external substrate pool ($V_{ex}[S]_{ex}$) due to the very low internal volume of the active proteoliposomes (V_{in}). Under these conditions a more reliable signal can be monitored if the internal substrate pool is labelled allowing measurements of the export of radioactivity after addition of unlabelled substrate to the incubation medium.

We were very careful to correct the observed rates of the carnitine/carnitine or carnitine/acylcarnitine exchange for the contribution of the uniport by subtracting the rate of the uniport ($V_{observed} - V_{uniport} = V_{exchange}$). For the forward-exchange measurements no corrections were necessary because of the low internal volume of active liposomes. In the case of the backward-exchange measurements ($v'_{observed} - v'_{uniport} = v'_{exchange}$), small corrections were necessary only when the ratio between the internal and external concentrations was very high, because the rate of uniport is at least one order of magnitude lower than the rate of exchange [13]. The rate of the uniport is calculated according to the equation:

$$v'_{uniport} = v'_{uniport_0}(1 - F)$$

where $v'_{uniport}$ is the apparent rate of uniport under the actual experimental conditions (internal concentration z and external concentration x), $v'_{uniport_0}$ is the apparent rate of uniport in the absence of external substrate and F is the ratio between the rate of uniport at external substrate concentration x and that in the absence of external substrate. $v'_{uniport_0}$ is easily measurable from the efflux of label inside the liposomes (see above) and F is obtained from the equation $F = (dpm_x - dpm_0)/dpm_x$. dpm_x and dpm_0 are measured as

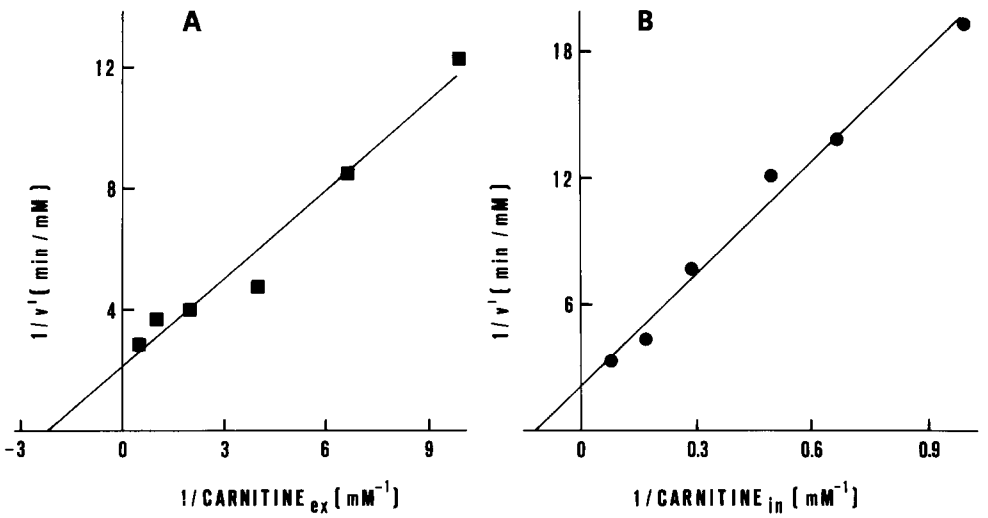

Fig. 1. K_m values of the reconstituted carnitine carrier for carnitine measured at the external (A) and internal (B) side of the liposomal membrane. Lineweaver-Burk plots were obtained from backward-exchange measurements of rate constant, k, under variation of external (A) or internal (B) carnitine. For definition of v', see Materials and Methods. In (A) the concentration of internal carnitine was 13 mM and in (B) the concentration of external carnitine was 10 mM.

follows: parallel samples of proteoliposomes containing unlabelled carnitine at internal concentration z are preincubated with carnitine at external concentration $= x$ or at external substrate = 0. After 20 min each sample, treated with mersalyl as described above, is passed through Sephadex G-75 column and then incubated for 60 min with 0.1 mM [^{3}H]carnitine plus 0.2 mM DTE (see also above). dpm_x and dpm_0 represent the radioactivity taken up by the proteoliposomes preincubated with external carnitine concentration x or 0, respectively. As an example, in the experiment illustrated in Fig. 4, only two measurements had to be corrected for the uniport contribution, i.e., the observed rate in the presence of 5.99 mM and 1.69 mM internal carnitine at 0.2 mM external carnitine. The corrected $1/v'$ values were 7.15 instead of 6.44 min/mM (5.99 mM internal carnitine) and 14.35 instead of 13.80 min/mM (1.69 mM internal carnitine), respectively.

Other methods. Protein was determined by the Lowry method modified for the presence of Triton [23]. All the samples used for protein determination were subjected to acetone precipitation and redissolved in 1% SDS [11].

Results

Determination of external and internal transport affinity

The transport affinity for carnitine on the external membrane surface of the reconstituted carnitine carrier was determined by the forward exchange method [12]. The analysis of the inward-facing site, which has not yet been described for the reconstituted carnitine carrier, can in principle be carried out by using the backward-exchange method, which allows antiport to be measured even at low internal substrate concentrations (see Materials and Methods). In order to check whether the backward-exchange technique is applicable also to the reconstituted carnitine carrier, the K_m of carnitine on the external side, previously determined by forward-exchange experiments [12], was measured by the backward exchange procedure under the conditions of Ref. 12, i.e., in the presence of 13 mM internal carnitine and external carnitine ranging from 0.1 to 2 mM (Fig. 1A). The K_m obtained from this and other experiments (0.45 ± 0.08 mM) agreed well with the value of 0.51 mM reported in Ref. 12 (Table I), indicating that the applied procedure was experimentally appropriate.

The intraliposomal K_m for carnitine/carnitine exchange was therefore determined by the backward exchange method. As shown in Fig. 1B, a linear reciprocal plot was obtained over a range of 1–13 mM internal substrate, keeping external carnitine constant at 10 mM, i.e., 20 times its K_m. From five experiments of this type a K_m of 8.7 ± 2.0 mM (Table I) was calculated for the internal binding site of the carrier. Since the external K_m (Fig. 1A) appeared to be several times lower than the internal K_m (Fig. 1B), we also looked for a lower K_m on the inside by varying internal carnitine from 0.14 to 4 mM at a constant external concentration of 10 mM carnitine. A similar internal K_m (9.0 ± 1.5 mM in three experiments) was also extrapolated in this case, thus excluding the presence of a second inward-facing binding site with a higher affinity for carnitine. In other experiments the dependence of the exchange rate on internal substrate concentration was investigated at a constant external concentration of carnitine lower than 10 mM. It was found that the K_m of carnitine on the internal side was 5.9 ± 1.9 mM (three experiments) in the presence of 1 mM external carnitine and 2.2 ± 0.4 mM (five experiments) in the absence of external carnitine.

Since for methodological reasons (see Materials and Methods) the forward-exchange technique can be applied to measure internal transport affinities when the internal substrate concentrations are not too low (≥ 1 mM) and the external concentrations not too high (≤ 1

TABLE I

External and internal K_m values of carnitine for the reconstituted carnitine carrier

Transport affinities were determined by the forward and by the backward exchange method (see Materials and Methods). The values are means ± S.E. (n). n.d., not determined. K_m values at finite countersubstrate concentration were obtained from Lineweaver-Burk plots. 'Concentration-independent' K_m values (i.e., at infinite countersubstrate concentration) were obtained by extrapolation from replots of primary plots of two-substrate analysis (Figs. 2C and 2D).

Substrate	Membrane side	Countersubstrate	K_m (mM)	
			forward exchange	backward exchange
Carnitine	external	carnitine (13 mM)	0.51 ± 0.14 * (20)	0.45 ± 0.08 (3)
	internal	carnitine (10 mM)	n.d.	8.7 ± 2.0 (8)
	internal	carnitine (1 mM)	5.8 ± 1.3 (3)	5.9 ± 1.9 (3)
	internal	none		2.2 ± 0.4 (5)
	external	carnitine (∞)	1.1 ± 0.1 (4)	1.1 ± 0.2 (3)
	internal	carnitine (∞)	10.0 ± 0.8 (4)	11.0 ± 2.1 (3)

* From Ref. 12.

mM), we used this independent technique to determine the internal K_m under these experimental conditions. The results obtained (internal $K_m = 5.8 \pm 1.3$ mM in three experiments, in the presence of 1–13 mM internal carnitine and 1 mM external carnitine) confirmed the presence of a single binding site on the internal side with a rather low affinity for carnitine.

As a general objection against this type of experiments it has been argued that this high internal K_m value could be due to artefacts, e.g., the markedly different curvature of the membrane. For this reason the proteoliposomes, after formation, were frozen, thawed and sonicated in order to scramble the orientation of the carrier molecules. After this procedure, we expected to find the high K_m also on the external face. Fig. 2 shows an Eadie-Hofstee plot of the transport rate as a function of external carnitine (0.1–8.0 mM) before and after freeze/thaw/sonication of the proteoliposomes (20 mM carnitine inside). A non-linear dependence became evident after sonication of proteoliposomes (Fig. 2B). The two different K_m values observed, 1.0 mM and 9.0 mM, corresponded to the previously determined external and internal K_m, respectively. The low-affinity and high-affinity kinetic components contributed to V_{max} in a ratio of approximately 4:1. Obviously the orientation of the membrane-inserted carrier molecules was substantially inverted by the freeze/thaw/sonication treatment.

Kinetic mechanism of exchange

The counterexchange of substrates catalyzed by an antiport carrier follows one of the two basically different two-substrate mechanisms, the ping-pong or the sequential type [24–27]. In the ping-pong mechanism, the first substrate (transported from side a to side b) is released before the second substrate binds to the carrier at side b. Thus only one binding site exists which is alternatively exposed to each side of the membrane. The sequential mechanism, on the other hand, involves the binding of the two substrates at the same time leading to the formation of a ternary complex with the carrier protein, before translocation takes place. In order to discriminate between these two possibilities for the reconstituted carnitine carrier, the exchange activity had to be analyzed by varying in a single experiment both external and internal substrate concentrations in the K_m range. Fig. 3 shows the results of such a bireactant initial-velocity study (two-substrate analysis) of the carnitine/carnitine homoexchange reaction, carried out by applying the forward-exchange method. When the kinetic data were analyzed in Lineweaver-Burk plots, showing the dependence of the transport rate on external carnitine (0.17–1 mM) at three different internal concentrations (4, 7 and 12 mM), a parallel pattern of straight lines was obtained (Fig. 3A). In other words the ratio of K_m/V_{max} was virtually constant as the internal substrate increased. If the same data were plotted as a function of the internal carnitine concentration (Fig. 3B), a similar pattern was obtained. This parallel pattern, in contrast to the intersecting pattern of a sequential reaction, indicates that the carnitine/carnitine exchange reaction follows a ping-pong mechanism [24–27]. Clearly, variation of the substrate concentration in one compartment strongly influences the apparent transport affinity for the respective countersubstrate in the other compartment. This mutual dependence could be quantitatively analyzed in secondary plots of the slopes and ordinate intercepts of Figs. 3A and 3B vs. the reciprocal concentration of the respective opposite substrate (Figs. 3C

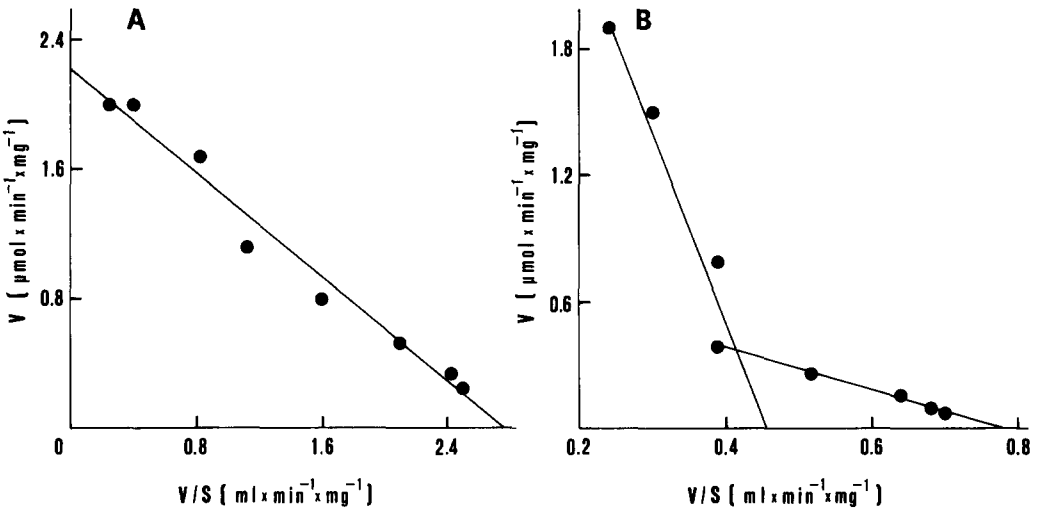

Fig. 2. Reshuffling of internal and external binding sites of the reconstituted carnitine carrier after freeze/thaw/sonication of proteoliposomes. K_m values for carnitine were measured at the external membrane side by the forward-exchange method before (A) and after (B) freeze/thaw/sonication of the proteoliposomes. The carnitine concentrations were 20 mM inside and 0.1–8.0 mM outside. The K_m values were calculated from the slope of the Eadie-Hofstee plots. *S*, external carnitine.

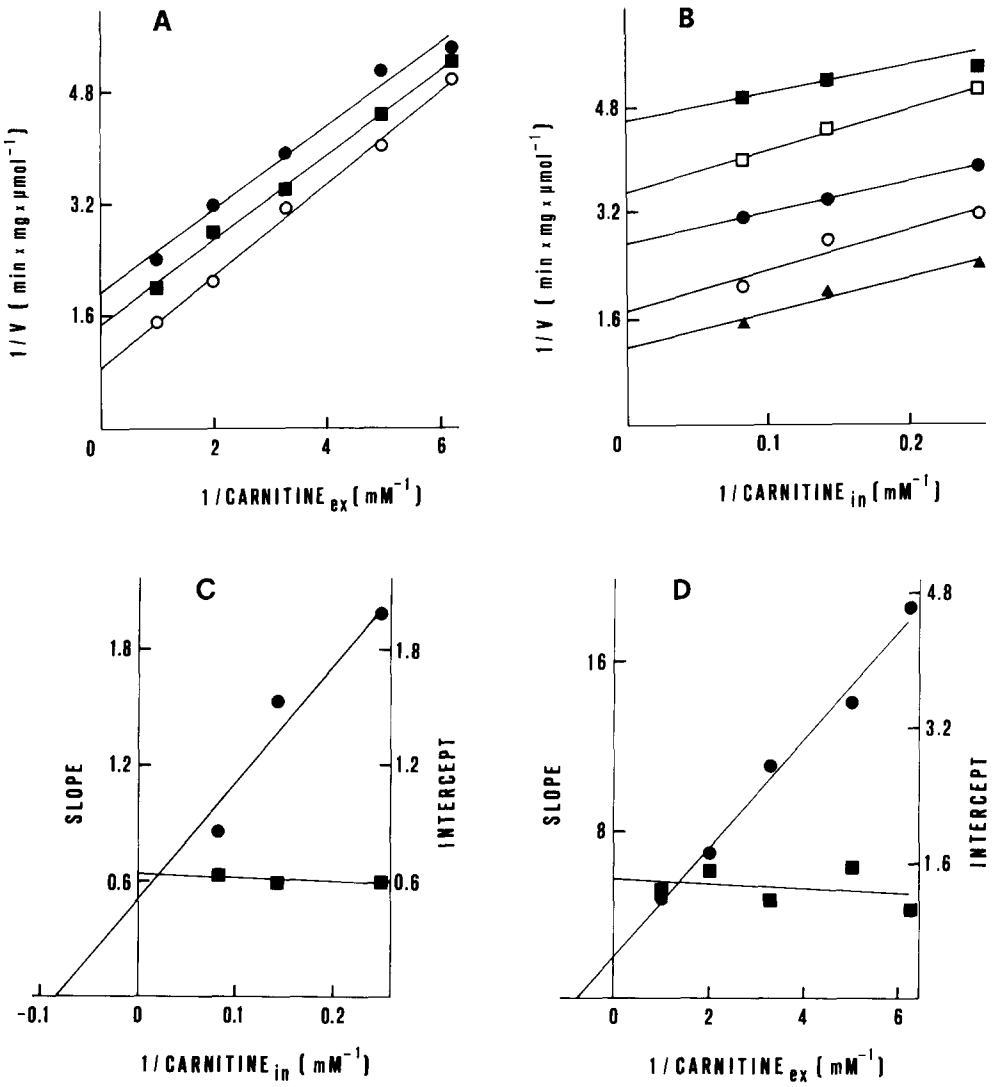

Fig. 3. Two-substrate analysis by the forward-exchange method of the carnitine/carnitine exchange reaction catalyzed by the reconstituted carnitine carrier. Lineweaver-Burk plots showing the dependence of exchange activity on external (A) and internal (B) carnitine. The concentrations of the countersubstrates were as follows: (A) 4 (●), 7 (■) and 12 (○) mM internal carnitine; (B) 0.17 (■), 0.2 (□), 0.3 (●), 0.5 (○) and 2 (▲) mM external carnitine. (C) and (D) Slope (■) and intercept (●) replots of the primary plots A and B, respectively.

and 3D). Figs. 3C and 3D clearly show that the ratio of K_m/V_{max} did not significantly change on changing the concentration of the countersubstrate. Furthermore, from the intercepts of the secondary plots K_m values could be derived that are extrapolated to infinite concentration of the respective second substrate [24]. These so-called 'concentration-independent' K_m values, 1.2 mM for external carnitine (1.1 ± 0.1 mM in four experiments) and 11.6 mM for internal carnitine (10.0 ± 0.8 mM in four experiments), were higher than those measured at finite substrate concentration (see above; Table I).

In order to corroborate this unexpected result, in the experiment reported in Fig. 4, the backward-exchange technique was applied. Internal carnitine was varied from 0.6 to 6 mM and external carnitine from 0.2 to 5 mM. A parallel pattern of straight lines was also obtained in this case, i.e., the ratio of K_m/V_{max} remained constant as countersubstrate concentration rose. On applying secondary plots (not shown) the 'concentration-independent' K_m values for internal and external carnitine were determined to be 11.5 mM (11.0 ± 2.1 mM in three experiments) and 1.2 mM (1.1 ± 0.2 mM in three experiments) respectively, i.e., very close to those obtained by the forward exchange method (see Table I). Thus, these findings substantiate the results obtained above by an independent method, which allowed the internal carnitine to be decreased to levels clearly below the internal K_m.

In order to establish whether ping-pong kinetics is in fact the general transport mechanism of the reconstituted carnitine carrier, it was important to determine whether this particular mechanism also holds true for the carnitine/acylcarnitine heteroexchange reaction.

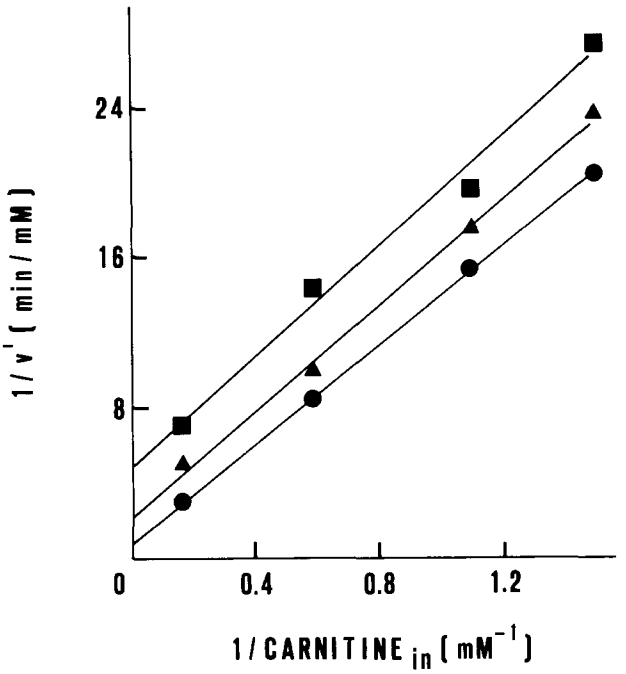

Fig. 4. Two-substrate analysis by the backward-exchange method of the carnitine/carnitine exchange reaction catalyzed by the reconstituted carnitine carrier. Lineweaver-Burk plot showing the dependence of exchange activity on internal carnitine. The concentrations of external carnitine were 0.2 (■), 0.7 (▲) and 5 (●) mM. Apparent exchange rates v' were calculated as described in Materials and Methods for the backward-exchange technique.

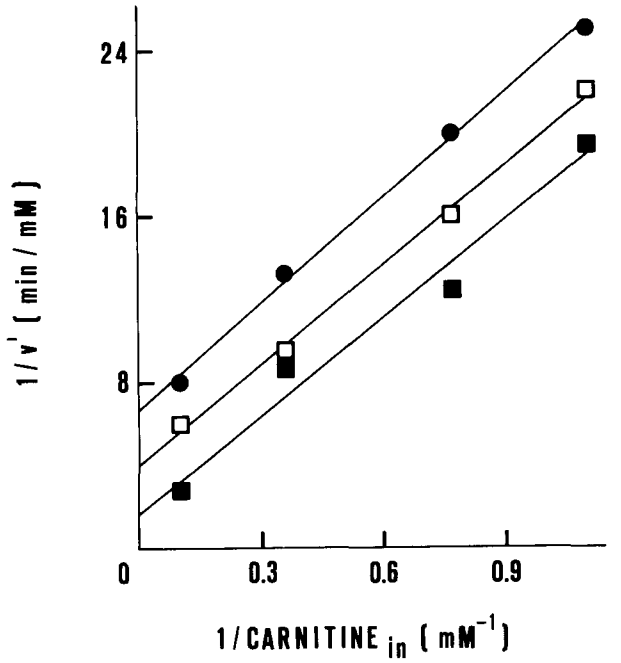

Fig. 5. Two-substrate analysis by the backward-exchange method of the propionylcarnitine/carnitine heterologous exchange reaction catalyzed by the reconstituted carnitine carrier. Lineweaver-Burk plot showing the dependence of exchange activity on internal carnitine. The concentrations of external propionylcarntine were 0.2 (●), 0.5 (□) and 5 (■) mM. Apparent exchange rates v' were calculated as described in Materials and Methods for the efflux procedure.

For this reason the carnitine/propionylcarnitine exchange reaction was investigated by the backward-exchange method (Fig. 5). The internal carnitine was varied from 0.9 to 10 mM and the external propionylcarnitine from 0.2 to 5 mM. Again a kinetic pattern of the ping-pong type was observed, demonstrating that the reaction mechanism was not altered when a different substrate was translocated. The 'concentration-independent' K_m values for internal carnitine and for external propionylcarnitine were 9.4 and 0.6 mM, respectively. A very similar kinetic pattern was also obtained when acetylcarnitine or valeroylcarnitine were used as countersubstrates instead of propionylcarnitine (not shown).

Discussion

It is a fundamental prerequisite for detailed kinetic studies of carriers in reconstituted systems to establish the orientation of the inserted translocator protein. Since antibodies or side specific inhibitors were not available for the carnitine carrier, we used a kinetic method for discrimination of the two faces of the reconstituted carrier protein. This method has successfully been applied before for several other mitochondrial carrier proteins in the reconstituted state [1]. We determined both the internal and external half-saturation constant (transport affinity) K_m for carnitine binding to the reconstituted carnitine carrier by application of two different kinetic methods, i.e., forward-exchange and backward-exchange. The two methods led to identical K_m values, which were clearly asymmetric, i.e., the internal K_m was always significantly higher than that on the outside (Figs. 1 and 2A). In view of the kinetic mechanism which we determined for the reconstituted carnitine carrier (see below), an objection may be put forward. Due to this particular mechanism, the substrate affinity on one side strongly depends on the concentration of the countersubstrate on the other side. The detailed kinetic analysis, however, resulted in so-called 'concentration-independent' K_m values for carnitine transport which also turned out to be significantly different for the two sides by one order of magnitude (about 1 mM for external and 10 mM for the internal side). As additional proof of the unidirectional orientation of the carnitine carrier in the proteoliposomal membrane we scrambled the orientation of the inserted carrier molecules by a freeze/thaw/sonication procedure. Consequently, this led to the experimental observation of two different carrier populations showing the two different K_m values on one single side of the membrane.

For the external K_m of carnitine in intact mitochondria, values between 0.3 and 2 mM have been determined [6,7,9] which agree well with the K_m we

measured on the external liposomal face. Since the internal K_m in mitochondria is not known, we suggest that the reconstituted carnitine carrier is inserted in right-side-out orientation. Preferential right-side-out orientation after reconstitution has been reported for several mitochondrial carriers, e.g., the aspartate/glutamate [28], the tricarboxylate [29], the dicarboxylate [30], and the phosphate carrier [31], as well as the uncoupling protein [32]. If indeed in intact mitochondria the K_m for carnitine on the matrix side is also much higher than that on the cytosolic side, our results have important physiological implications. The intramitocondrial level of carnitine most likely regulates fatty acid translocation into the mitochondria and probably the overall process of fatty acid oxidation, since the carnitine concentration in the matrix [7–9] is lower than its affinity to the carnitine carrier on the internal side. Due to the particular type of mechanism, increasing internal concentrations of carnitine lower the external affinity for acylcarnitine. Under these conditions (high internal carnitine), however, net efflux of carnitine mediated by the uniport mode of the carrier may become important too.

An obvious objection to the argument for different apparent transport affinities on the two sides of the membrane may be put forward in view of the mechanism concluded to be valid for the carnitine carrier (see below). In a ping-pong mechanism, in fact only one binding site is assumed, alternatively exposed to the two sides of the membrane. It is, however, clear that this does not mean that an identical ensemble of amino acids is responsible for the two alternative faces of the binding site. Different substrate affinities have also been found in the case of other ping-pong type carriers [33].

The bisubstrate initial-velocity studies which we used to elucidate the kinetic mechanism of the reconstituted carnitine carrier led to a clear answer. The pattern of parallel lines in double reciprocal plots for both the homologous carnitine/carnitine exchange and the heterologous carnitine/acylcarnitine exchange indicates a ping-pong type of transport mechanism. In contrast to sequential mechanism, the ratio of K_m/V_{max} stayed constant upon variation of the countersubstrate concentration. A ping-pong type of mechanism involves only binary carrier substrate complexes, according to the assumption of a single binding site, alternatively exposed to the two sites of the membrane, whereas a sequential mechanism would include a ternary complex of two substrates with the carrier protein. The kinetic mechanism which we deduced for the reconstituted carnitine carrier agrees with earlier observations in intact mitochondria [9] that the K_m for carnitine on the cytosolic side increases from 0.5 to 0.9 mM on raising the matrix carnitine content. It possibly also accounts for the relatively wide range of K_m values observed for external carnitine in intact mitochondria [6,7,9].

The other mitochondrial carrier proteins kinetically characterized in reconstituted systems so far, have all been found to function according to a sequential type of mechanism, involving a ternary complex within the catalytic cycle [1]. This has been proven for the aspartate/glutamate [34], the oxoglutarate [22], the citrate [29], the dicarboxylate [30], as well as the phosphate carrier (Stappen, R. and Krämer, R., unpublished data). With respect to the structural aspect, based on the primary sequence of four mitochondrial translocators, a common structural family of these transport proteins was deduced [15–17]. Based on the 'common molecular mass' of mitochondrial carriers of about 30–34 kDa on SDS-gels, the carnitine carrier falls into the same family [11]. Clearly, knowledge of its primary structure is needed to answer the question whether or not this assumption is in fact valid.

The presence of both a ping-pong type and a sequential type of mechanism within the same structural family is not completely unexpected. The same observation was made in the family of proteins related to the erythrocyte anion carrier [35]. These considerations directly lead to a more general view of the mechanism of the carnitine carrier in comparison to the other mitochondrial translocators. The majority of them catalyze antiport, some mediate H^+-compensated unidirectional substrate flux. However, at least in the case of the phosphate carrier which falls into the latter class, experimental evidence has been obtained that this carrier in fact also functions in a phosphate/OH^- antiport mode (Stappen, R. and Krämer, R., unpublished data). The only mitochondrial translocators mediating real substrate uniport are the uncoupling protein of brown fat mitochondria and the carnitine carrier. However, for the uncoupling protein, in contrast to the carnitine carrier, the uniport mode is the exclusive transport mode. Since the carnitine carrier in the exchange mode functions in a ping-pong type of mechanism, the uniport mode necessarily involves a reorientation step of the unloaded carrier form (C). For the other mitochondrial carriers, except the uncoupling protein, this function would actually mean a slippage reaction, i.e., a step within the catalytic cycle which is normally forbidden by energetic rules [36]. Consequently, the activation energy barrier for a conformational change of this carrier species (C) must be much lower in the case of the carnitine carrier. On the basis of these considerations it seems to be appropriate that in a carrier system like the carnitine carrier where the uniport function is essential, the common sequential mechanism has been changed into a ping-pong type of mechanism. Due to a simpler construction principle using only binary complexes (CS), slippage reactions involving 'mobility' of the unloaded form (C) can pre-

sumably be achieved more easily as compared to sequential mechanisms where the ternary complex (CS_2) is the transport intermediate.

The basic relationship to the common functional family of mitochondrial carrier, however, is closer than may be thought in view of the different mechanism observed. It has previously been shown that the carnitine carrier can be converted to an 'unphysiological' mode of unspecific uniport by modification of particular cysteine residues [14]. This property seems to be characteristic for mitochondrial carrier proteins [1] and has been found for the aspartate/glutamate and the ADP/ATP carriers [37], the phosphate carrier [31], as well as the oxoglutarate and the tricarboxylate carriers (Indiveri, C., Bisaccia, F. and Palmieri, F., unpublished data), besides the carnitine carrier [14]. Thus, in spite of the different kinetic mechanism, this observation again convincingly argues in favour of the carnitine carrier being a member of the common family of mitochondrial carrier proteins.

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