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Argininosuccinate Synthetase and Argininosuccinate Lyase Are Localized Around Mitochondria: An Immunocytochemical Study

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Abstract Argininosuccinate synthetase and argininosuccinate lyase are soluble cytoplasmic enzymes of the urea cycle. Previous biochemical studies using permeabilized hepatocytes showed that these enzymes are organized in situ, and function as if they are located next to the outer membrane of mitochondria. We have now confirmed and extended those observations in intact liver by means of immunocytochemistry at the electron microscope level. Morphometric analysis of the electron micrographs shows that argininosuccinate synthetase and argininosuccinate lyase are located in the immediate vicinity of the mitochondria, predominantly next to the cytoplasmic surface of the outer membrane. Some immuno-specific protein is also observed in the endoplasmic reticulum in the immediate vicinity of the mitochondria. These results support our previous biochemical findings, and additionally suggest that virtually all of the argininosuccinate synthetase and argininosuccinate lyase of the liver parenchymal cell are located just outside the mitochondria. © 1996 Wiley-Liss, Inc.

Key words: urea cycle, liver, enzyme organization, cell organization, electron microscopy, immunogold, immunocytochemistry

The urea cycle of mammalian liver consists of five enzymes, of which the first two are exclusively in the mitochondrial matrix [Clarke, 1976; Gamble and Lehninger, 1973], and the next three are cytoplasmic [Ratner, 1976]; the reactions (not balanced for charge) are shown in Figure 1.

All of these enzymes are soluble, i.e., they are released into solution in the absence of detergent when the cells or organelles are disrupted. The behavior of the individual enzymes in solution, however, cannot account for several aspects of the operation of the pathway in situ [Cohen et al., 1980, 1982, 1985, 1987, 1992; Cheung et al., 1989]. In previous biochemical work using intact and permeabilized isolated mitochondria and permeabilized hepatocytes, it was shown that the pathway of urea synthesis is organized sequentially so that intermediates are channeled at each step [Cohen et al., 1987, 1992; Cheung et al., 1989]. That is, endogenously generated intermediates are kept within the pathway, and are not free to mix in the bulk aqueous phase of the cell. This occurs even though the enzymes are in two separate cell compartments. The demonstration that citrulline is channeled from ornithine transcarbamylase in the mitochondrial matrix across the mitochondrial membranes to argininosuccinate synthetase (ASS) in the cytoplasm, and that tight channeling of intermediates occurs between each of the cytoplasmic members of the pathway, provided strong evidence that these cytoplasmic enzymes are located next to the outer mitochondrial membrane [Cheung et al., 1989].

These studies, which utilized isotope dilution techniques and kinetic analyses, did not provide information regarding the amount of each of the cytoplasmic enzymes that is localized next to the mitochondria. Moreover, since the experiments were done with permeabilized hepatocytes, one could argue that they may not accurately reflect the organization of this pathway in the intact

Abbreviations used: ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase.

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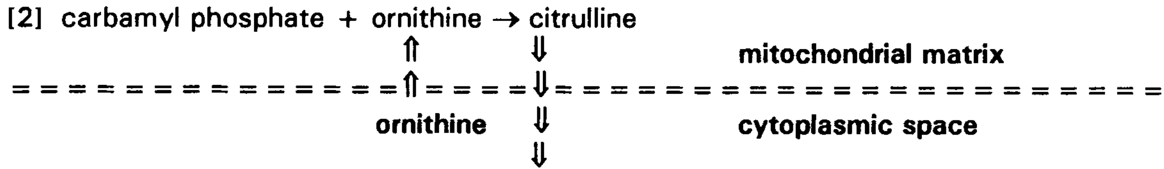

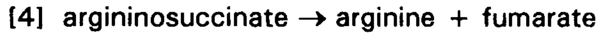

Fig. 1. The pathway of urea synthesis in mammalian liver. Reactions [1] through [5] are catalyzed, respectively, by carbamyl phosphate synthetase (ammonia) (EC 6.3.4.16), ornithine transcarbamylase (EC 2.1.3.3), argininosuccinate synthetase (EC 6.3.4.5), argininosuccinate lyase (EC 4.3.2.1), and arginase (EC 3.5.3.1). The dashed lines represent the mitochondrial membranes.

cell. In this paper we present immunocytochemical results obtained in intact liver which support the previous findings, and show that virtually all of the argininosuccinate synthetase and argininosuccinate lyase (ASL) are organized around the mitochondria in intact cells.

MATERIALS AND METHODS

Male Sprague-Dawley rats (200–220 g) were obtained from Simonsen Laboratories, Gilroy, CA. Rabbit antisera raised against human ASS and ASL were a generous gift from Dr. William E. O'Brien. The cross-reactivity of the antibodies with the rat enzymes was examined on immunoblots of various preparations from normal rat liver. These included hepatocytes and permeabilized hepatocytes [Cheung et al., 1989], both of which are capable of de novo urea synthesis; nude hepatocytes [Katz and Wals, 1985], which have lost most of their soluble cytoplasmic enzymes; isolated mitochondria [Cohen et al., 1985]; and tissue homogenates. Aliquots of each of these preparations were denatured by boiling for 3 min in denaturing buffer [Laemmli, 1970]. The samples were electrophoresed on 0.1% SDS/9% acrylamide slab gels as described [Laemmli, 1970]. The proteins were electrophoretically transferred to nitrocellulose membranes, using an ABN semi-dry transfer apparatus, and immunoblotted according to the Bio-Rad (Hercules, CA) Immun-Blot protocol. The incubation time for the primary antibodies (diluted 1:1,000) was 2 h.

The liver used for electron microscopy was from an adult male Sprague-Dawley rat that had been fed a diet containing 60% protein for 8 days. It has been shown that levels of ASS and ASL proteins are increased about 2-fold when rats are transferred from a normal diet of 23% protein, to one containing 60% protein [Schimke, 1962]. One millimeter slices of liver were fixed in 4% paraformaldehyde/20 mM ethylacetimidate in 0.1 M phosphate buffer, pH 10, for 7 min, and then in 4% paraformaldehyde/0.1% glutaraldehyde in phosphate buffer, pH 7.2, for 1 h [Geiger et al., 1981]. They were rinsed, cut into 1 mm cubes, dehydrated in graded alcohols, and embedded in LR white resin. Thin sections were picked up on parlodion-filmed nickel grids, etched for 20 min in saturated sodium metaperiodate (0.5 mg/ml), rinsed, blocked for 1 h in 5% globulin-free BSA, and exposed overnight at 4°C, either to a 1:200 dilution of antiserum to ASS or ASL, or to rabbit IgG as a control. The buffer for diluting the antibodies and rinsing was 50 mM Tris/HCl, pH 7.2, 250 mM NaCl, 0.1% BSA. After rinsing (6 × 2 min), the grids were exposed to goat anti-rabbit/biotin, 2 μg/ml, for 1 h, rinsed, treated with streptavidin/gold (10 nm), 1:100, for 1 h, rinsed, and stained for 5 min in 3.5% uranyl acetate. Electron micrographs were taken on a JEOL 1200CX microscope.

The electron micrographs were quantitated by morphometric analysis. The relative amounts of mitochondrial matrix, mitochondrial outer membrane, endoplasmic reticulum, and nuclei

were determined by the point counting method [Weibel and Bolender, 1973], and the total numbers of gold particles and their location were scored. Point counting was done by overlaying the micrographs with a regular grid of intersecting lines, and counting the number of intersections that fell over each of the recognizable structures of interest. The "density" of each structure in a micrograph was the number of intersections which fell on that structure, expressed as a percentage of the total number of intersections. In the same manner, the total number of gold particles was counted, and the number over each of the recognizable structures was scored; this was done under a magnifying lens. The "relative frequency" of gold over each structure is equal to the number of particles over that structure as a percentage of the total gold particles on the micrograph. Random background gold particles generate a ratio of relative frequency to density of 1.0 or lower. Ratios greater than 1.0 indicate that there is a preferential association of the signal with the structure in question [Weibel and Bolender, 1973].

RESULTS

Because the antibodies to be used for immunocytochemistry had been raised against ASS and ASL from human liver, their cross-reactivity with the rat proteins was ascertained on immunoblots of SDS gels of preparations from rat liver.

To test the antiserum to ASS, preparations of intact, permeabilized, and nude hepatocytes, and mitochondria were electrophoresed. Permeabilized cells, which are capable of de novo urea synthesis, were examined because they were the experimental material used in previous biochemical studies showing that ASS and ASL appear to be located near the mitochondrial membrane [Cheung et al., 1989]. Nude cells, which are prepared by digitonin treatment of isolated hepatocytes, were of interest because they lack the plasma membrane and lose most of their soluble cytoplasmic enzymes, while retaining most organelles and the cytoskeleton [Katz and Wals, 1985]. We have observed that unlike permeabilized cells, nude cells cannot synthesize urea de novo [Cohen et al., unpublished data]; citrulline accumulates instead, indicating that only the mitochondrial portion of the urea cycle remains intact. Isolated mitochondria were compared with these cell samples. Because ASS is readily released into the soluble phase when cells are disrupted, preparations of isolated mitochondria would not be expected to contain significant quantities of this enzyme, even though the latter was shown to interact directly with mitochondria in situ in permeabilized cells [Cheung et al., 1989]. The results are shown in Figure 2A. Lane 2 contained 20 μg of protein from intact hepatocytes. The quantities of protein loaded in lanes 3 (permeabilized cells) and 4 (nude cells) were those estimated to represent approximately the same amount of original cell material as that in lane 2. The amount of mitochondrial protein loaded in lane 1, however, was the quantity estimated to come from at least twice as much intact cell material as that represented in lane 2. Exposure of the blot to antiserum to ASS produced a single band in the lanes containing extracts of mitochondria (lane 1), or of intact (lane 2) or permeabilized (lane 3) hepatocytes. Despite the relatively large quantity of mitochondrial protein loaded, the band observed in the mitochondrial sample was extremely faint, showing that only a very small amount of ASS remains associated with these organelles or with the fragments of endoplasmic reticulum which remain attached to isolated mitochondria [Katz et al., 1983]. In contrast to permeabilized cells (lane 3), nude cells (lane 4) appear to have lost all of their ASS.

The antiserum to ASL was tested against liver homogenate (Fig. 2B). A single band was observed, which increased in intensity as the amount of sample was increased. For both antisera, comparison of the location of the bands on the immunoblots with those of blotted prestained standards run on the same gels showed that the immunospecific proteins were of the size expected for ASS and ASL, i.e., 45 and 52 kDa, respectively [Ratner, 1976] (not shown).

Examination of the electron micrographs of sections treated with antibodies to either ASS or ASL (Fig. 3) showed that gold particles often occurred in the ER very close to, but not immediately adjacent to, the mitochondrial membrane. Gold particles which were either directly over, or immediately adjacent to or within about 30 nm of the cytoplasmic side of the mitochondrial membranes, were scored as "mitochondrial membrane-associated." This location of the gold particles was assessed using a magnifying lens, and every effort was made to avoid bias; only those particles which clearly fit the above definition were scored as such. Analysis of ten micrographs for each of the two enzymes showed

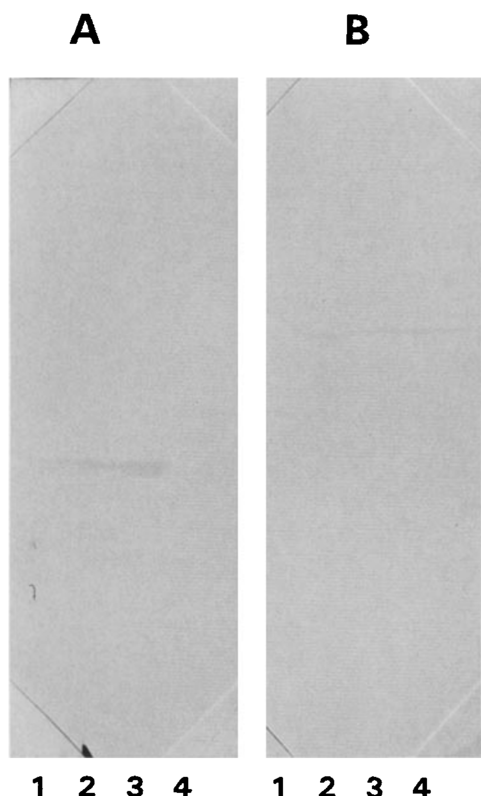

Fig. 2. Specificity of anti-ASS and anti-ASL. Immunoblots of 0.1% SDS/9% acrylamide gels of various preparations from normal rat liver are shown for ASS (**A**) and ASL (**B**). For A the samples were: *lane 1,* mitochondria (2.4 µg of protein); *lane 2,* isolated hepatocytes (20 µg of protein); *lanes 3* and *4,* permeabilized and nude hepatocytes, respectively (in amounts representing approximately the same quantity of original cells as the sample in lane 2). For B, lane 1 was blank, and lanes 2, 3, and 4 contained 17, 35, and 70 µg of protein from a liver homogenate, respectively. The gels were transferred to nitrocellulose membranes and immunoblotted as described in the text.

that mitochondrial membrane-associated gold particles were present at much higher frequency than would be expected from the density of the membranes. In contrast, the relative frequency of gold was the same as, or less than, the density of mitochondrial matrix, endoplasmic reticulum, and nuclei. For example, on a typical micrograph of an anti-ASS-treated section, 21.5% of the gold particles were associated with the mitochondrial membrane (n = 390), while only 13.0 percent of the total intersections fell over the outer membrane (n = 292). The ratio of the relative frequency of gold to the density of outer membrane was, therefore, 21.5:13.0, or 1.66. On the same micrograph the ratios for endoplasmic reticulum and mitochondrial matrix were 0.96 and 1.05, respectively. On the ten micrographs scored for each antibody, the mean enrichment ratio for outer membrane was 1.59 ± 0.07 for ASS and 1.77 ± 0.18 for ASL (M ± SEM). For all other structures, the mean ratios were ≤ 1.0, indicating that the appearance of gold particles over those structures was the result of random background. The ratios obtained from the micrographs of the anti-ASS-treated sections were: for mitochondrial matrix, 0.89 ± 0.05; endoplasmic reticulum, 0.91 ± 0.03; other (including nuclei), 1.02 ± 0.09. Ratios from the micrographs of the anti-ASL-treated sections were: matrix, 0.96 ± 0.04; reticulum, 0.90 ± 0.05; other (including nuclei), 0.97 ± 0.09. For sections treated with rabbit IgG instead of specific antibody, the number of gold particles was too low to conduct any quantitative analysis.

The mitochondria were not uniformly labeled with the immunogold. There were areas of the cells in which all or most of the mitochondria displayed gold particles, while within the same cell there were other areas in which none of the mitochondria had them. This suggests that the mitochondrial population of the liver parenchymal cell, and the proteins of the immediately surrounding cytoplasm, which may be associated with those organelles, may be heterogeneous in function, as suggested by previous work [Katz et al., 1983].

The electron micrographs shown in Figure 3 were taken from areas of the cells that were enriched in mitochondria. In rats fed a diet containing 60% protein for 8 days, the protein levels of all of the urea cycle enzymes are elevated compared to those in normal animals [Schimke, 1962], and the numbers of mitochondria are increased two- to three-fold [Didier et al., 1985] (Cohen et al., unpublished data). Consequently, there were relatively few areas of the cells that were devoid of mitochondria; when electron micrographs of these areas were examined, it was observed that there were fewer gold particles than in the mitochondria-rich areas; the enrichment ratio for such regions was always less than 1.0.

DISCUSSION

Previous biochemical studies from this laboratory provided strong evidence that the entire pathway of urea synthesis is organized in situ in such a way that intermediates are kept within the pathway. The functional organization of carbamyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC) was shown in studies of intact [Cohen et al., 1980, 1982] and

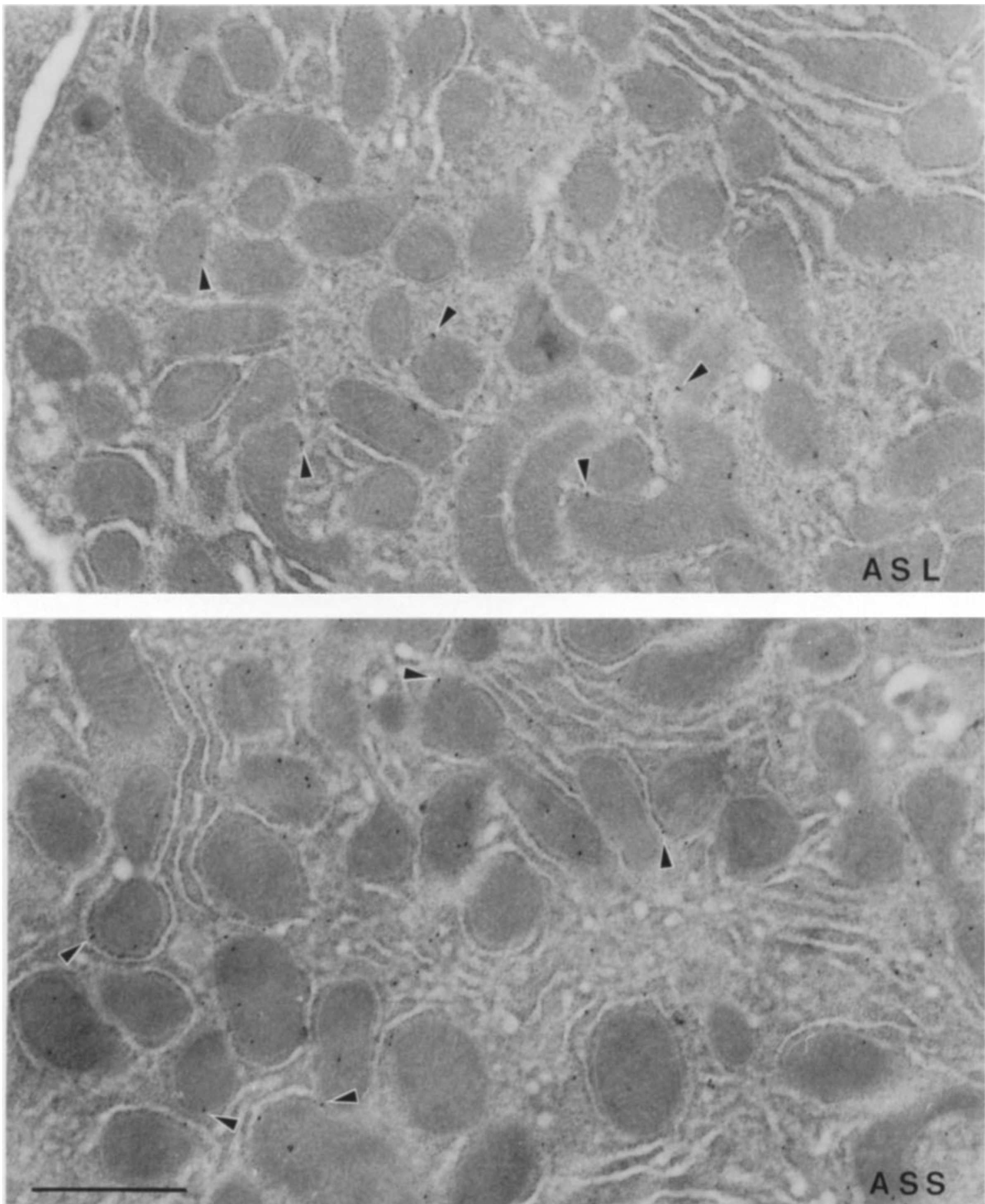

Fig. 3. Immunocytochemical localization of ASS and ASL. Immunogold labeling is shown for sections treated with antiserum to ASS or to ASL as described in the text. *Arrowheads* point to 10 nm gold next to the outside of the mitochondrial outer membrane. Scale bar, 1 μm.

permeabilized [Cohen et al., 1992] mitochondria. It was also demonstrated that extramitochondrial ornithine is channeled to OTC, and used preferentially for citrulline synthesis, even in the presence of a large preexisting pool of ornithine in the matrix [Cohen et al., 1987]. Using isotope dilution techniques in incubations of permeabilized hepatocytes, it was shown that endogenously synthesized arginine is very tightly channeled from ASL to arginase; a 1,000-fold excess of unlabeled arginine did not dilute labeled, endogenously synthesized arginine that was already within the pathway [Cheung et al., 1989]. In the same studies it was demonstrated that argininosuccinate is channeled from ASS to ASL. The channeling of these two intermediates showed that the three cytoplasmic enzymes are sequentially organized. Finally, the channeling of citrulline from its site of synthesis by OTC in the matrix to ASS in the cytoplasmic space was also demonstrated, indicating that ASS, and therefore ASL and arginase as well, must function at the mitochondrial membrane.

Although these studies provided strong and convincing direct evidence of the organization of this metabolic pathway in situ, one could argue that they merely represented artifactual conditions uniquely associated with the permeabilized cells, and might not reflect the situation in intact liver cells. For example, the composition of the incubation medium might have had critical effects on the behavior of the enzymes in the permeabilized cells. However, the observations reported in the present immunocytochemical work not only confirm the results of the biochemical experiments and their relevance to intact liver, but also suggest that most, if not all, of the cell ASS and ASL is localized near the mitochondria.

Taken together, the results of all of these studies support the schematic portrayal of the pathway of urea synthesis in Figure 4. This figure shows each enzyme as a multimer, based on biochemical data obtained for the purified enzymes [Clarke, 1976; Lusty et al., 1979; Ratner, 1976]. Also shown in Figure 4 is the channeling of carbamyl phosphate from CPS to OTC [Cohen et al., 1992]; of extramitochondrial ornithine to OTC in the matrix [Cohen et al., 1987]; of citrulline from OTC, across the membranes, to ASS in the cytoplasmic space [Cheung et al., 1989]; and of argininosuccinate and arginine [Cheung et al., 1989]. For simplicity the mitochondrial inner and outer membranes are depicted as a single line.

The specific protein-protein interactions involved in the organization of the urea cycle enzymes remain to be elucidated, and if elements of the endoplasmic reticulum or the cytoskeleton participate in that organization, they have not yet been identified. In addition, the mechanism whereby the cytoplasmic enzymes of the pathway are directed to their final destinations in the cell is unknown.

The successful immunocytochemical demonstration of ASS and ASL localization is especially gratifying because these two enzymes do not constitute a large portion of cytoplasmic proteins. ASS and ASL are not particularly abundant in normal liver, and even in animals transferred from a normal to a high-protein diet, the amount of these enzymes increases only about 2-fold [Schimke, 1962]. In this context, it was interesting to observe that the mitochondria of

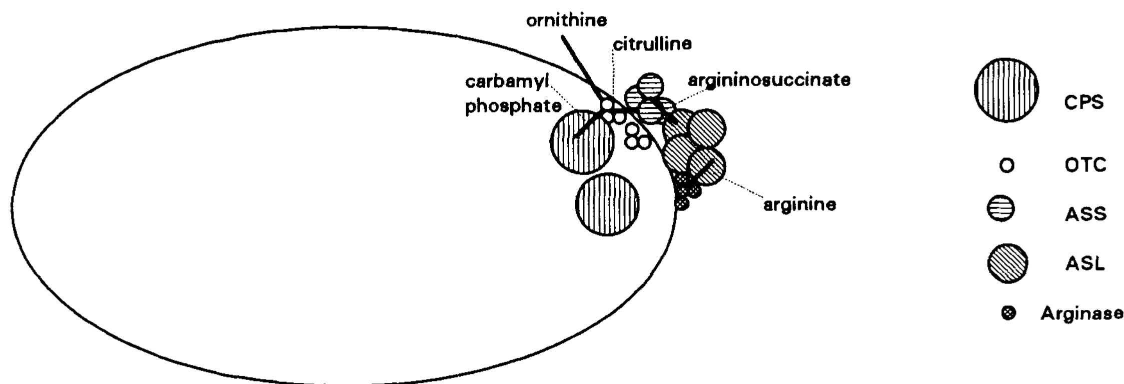

Fig. 4. Schematic diagram of the organization of the enzymes of urea synthesis. The diagram depicts a possible arrangement of the five enzymes within and around the mitochondrion, based on experimental evidence. The solid straight lines show extramitochondrial ornithine being channeled to OTC inside the matrix, and the channeling of other intermediates in the pathway between sequential enzymes. For simplicity, the mitochondrial inner and outer membranes are not shown separately.

any given cell were not uniformly labeled with gold particles, suggesting that there are differences in the content of the urea cycle enzymes in different regions of the cells. It has been determined that the enzymes of the urea cycle are present in all liver parenchymal cells except for a few perivenous cell layers which contain high specific activities of glutamine synthetase instead [Gebhardt et al., 1988]. There is no evidence for the enrichment of different metabolic pathways in various sites within the cytoplasmic space, although there may be gradients between the basal and apical portions of the cells. If such gradients do exist, there could be an accompanying heterogeneity of liver mitochondria. It was reported by Katz et al. [1983] that mitochondria sedimenting at 1,000–1,500*g* had higher specific activities of several matrix enzymes, and higher rates of oxygen uptake than mitochondria requiring 10,000*g* for sedimentation. Moreover, the ratio of the specific activities of OTC:CPS in the high-speed mitochondria is about twice that in the low-speed mitochondria [Katz et al., 1983] (Cohen et al., unpublished data). These data indicate that liver mitochondria are not homogeneous in their enzymatic content. The present immunocytochemical results indicate that there is heterogeneity in the enzymatic composition of different cytoplasmic regions. In addition, the non-uniform immunogold labeling of different mitochondria-rich regions of the cell with regard to ASS and ASL provides further evidence that liver mitochondria are heterogeneous, in that certain cytoplasmic enzymes associated with them or located in their immediate surroundings are not uniform throughout the cell.

The intracellular organization displayed by the enzymes of the urea cycle, all of which readily go into solution when cells are disrupted, is probably not unique to that metabolic pathway. On the contrary, there is abundant evidence suggesting that the organization of functionally related soluble enzymes in situ may be a property shared by other metabolic pathways [Srere, 1987]. Such organization may be required for the proper functioning of a given pathway in the complex intracellular environment, and may be important for the regulation of metabolic flux.

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