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# Kinetic properties of carbamoyl-phosphate synthase (ammonia) and ornithine carbamoyltransferase in permeabilized mitochondria

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Previous studies using intact rat liver mitochondria have shown that the soluble matrix enzymes carbamoyl-phosphate synthase (ammonia) (CPS) and ornithine carbamoyltransferase (OCT) display some kinetic properties which would not be observed if they were homogeneously distributed in the matrix. In the present work we have extended these studies, using toluene-treated mitochondria which are fully permeable to substrates and inhibitors, yet retain 90% of their soluble enzymes. The results provide evidence of functional organization of CPS and OCT *in situ*. The major findings are as follows. (1) The apparent $K_m$ values of matrix OCT for carbamoyl phosphate and ornithine are respectively 8 and 2 times those measured for the soluble enzyme. δ-*N*-Phosphonacetyl-L-ornithine inhibits OCT *in situ* less than in solution, especially when carbamoyl phosphate is synthesized in the mitochondria rather than added to the medium. (2) During citrulline synthesis from endogenously generated carbamoyl phosphate, the concentration of the latter in permeabilized mitochondria is more than 10 times that in the medium, although the mitochondria are freely permeable to added molecules of this size. (3) Endogenously formed carbamoyl phosphate is used preferentially by OCT *in situ*; addition of a 200-fold excess of unlabelled carbamoyl phosphate has little effect on the conversion of labelled endogenously formed carbamoyl phosphate into citrulline by matrix OCT. (4) The synthesis *de novo* of carbamoyl phosphate from $NH_3$, $HCO_3^-$ and ATPMg is the same in the presence and absence of ornithine. (5) Studies with co-immobilized CPS and OCT gave results concordant with some of the above observations and with previous ones with intact mitochondria.

## INTRODUCTION

Carbamoyl-phosphate synthase (ammonia) (CPS; EC 6.3.4.16) and ornithine carbamoyltransferase (OCT; EC 2.1.3.3), which catalyse the first two reactions of the pathway of urea synthesis, are located in the matrix of liver mitochondria. These soluble enzymes function as a coupled system in intact mitochondria, and their behaviour in the matrix is different from that observed in dilute solution. For example, CPS *in situ*, but not in solution, is greatly inhibited in the absence of ornithine, a substrate for OCT [1]. The binding sites of OCT appear to be relatively inaccessible to certain inhibitors, and to one of its substrates, carbamoyl phosphate, when they are added to intact mitochondria, even though these compounds enter the matrix [2]. In contrast, extramitochondrial ornithine is channelled to matrix OCT during citrulline synthesis in intact mitochondria, despite the presence of a large pre-existing pool of matrix ornithine [3].

These phenomena may be caused by the unique environment within the matrix, i.e. an extremely high protein concentration, of the order of 500 mg/ml [4], and a concomitant relative scarcity of water [5]. The mitochondrial inner-membrane surface is so extensive that it has been calculated that a major proportion of matrix proteins must be adjacent to it [6]. Such conditions are known to promote protein–protein interactions involving both soluble and membrane proteins that might not occur in dilute solutions [7–9]. CPS and OCT are abundant in the matrix; in liver mitochondria from rats fed on a normal diet, there are about 0.5 μmol of CPS [10,11] and 0.1 μmol of OCT [11] per ml of matrix water. Both CPS [12,13] and OCT [13,14] appear to be situated next to the inner membrane, and have been reported to remain loosely associated with inner-membrane fragments under some conditions [13].

Mitochondria treated with toluene are freely permeable to low-molecular-mass compounds such as substrates and inhibitors, while retaining most soluble enzymes [15]. As previously reported [16,17], these preparations are capable of carbamoyl phosphate and citrulline synthesis *de novo*. We have obtained further evidence of functional interactions between CPS and OCT *in situ*. The results show that matrix OCT uses endogenously formed carbamoyl phosphate preferentially. Some of these properties were reproduced by using purified CPS and OCT co-immobilized on an affinity medium.

## MATERIALS AND METHODS

### Animals and reagents

Male Sprague–Dawley rats weighing 150–250 g were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A.; they were fed *ad libitum* on standard laboratory chow. Reagents used for mitochondrial preparation and incubation were from Sigma Chemical Co., St. Louis, MO, U.S.A., except the following: polyethylene glycol 8000 (average $M_r$ 7000–9000) (PEG) was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A., BSA, essentially fatty-acid-free, was from Boehringer–Mannheim Biochemicals, Indianapolis, IN, U.S.A., [U-$^{14}$C]sucrose (sp. radioactivity 350 mCi/mmol), $^3H_2O$ (sp. radioactivity 100 mCi/ml), $NaH^{14}CO_3$ (sp. radioactivity 55 mCi/mmol) and [*carboxy*-$^{14}$C]inulin (sp. radioactivity 2.26 mCi/g) were from ICN, Irvine, CA, U.S.A., L-[*ureido*-$^{14}$C]citrulline (sp. radioactivity 57 mCi/

Abbreviations used: CPS, carbamoyl-phosphate synthase (ammonia) (EC 6.3.4.16); OCT, ornithine carbamoyltransferase (EC 2.1.3.3); PEG, polyethylene glycol 8000; PALO, δ-*N*-phosphonacetyl-L-ornithine.

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mmol), [$^{14}$C]carbamoyl phosphate (sp. radioactivity 5.1 mCi/mmol), and [*carboxy*-$^{14}$C]dextran (sp. radioactivity 1.24 mCi/g; $M_r$ 70000) were from Du Pont–New England Nuclear, Boston, MA, U.S.A. Hydrofluor was from National Diagnostics, Somerville, NJ, U.S.A., Dow Corning silicone 550 from William F. Nye, New Bedford, MA, U.S.A., and activated CH-Sepharose 4B from Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A. Bovine OCT (sp. activity 650 $\mu$mol/min per mg) was purified by the method of Marshall & Cohen [18], and rat CPS (sp. activity 1.5 $\mu$mol/min per mg) as described by Powers [19]. $\delta$-*N*-Phosphonacetyl-L-ornithine (PALO) was given by Dr. Evan E. Jones (North Carolina State University, Raleigh, NC, U.S.A.).

### Preparation and permeabilization of mitochondria

The rats were killed by cervical dislocation, and liver mitochondria were prepared in 0.3 M-mannitol/2 mM-Hepes/KOH, pH 7.4, exactly as described by Cohen *et al.* [20], except that the final mitochondrial pellet was resuspended to yield a protein concentration of at least 100 mg/ml. Protein was measured at once by the biuret method described by Jacobs *et al.* [21], with BSA as the standard, and then adjusted with additional mannitol/Hepes medium to 100 mg/ml.

The mitochondria were permeabilized with toluene by a modification, similar to that used by Lof *et al.* [16], of the method of Matlib *et al.* [15], as follows. The medium used (Medium X) was composed of 0.3 M-mannitol, 50 mM-Tris/HCl, pH 7.4, 2 mM-Hepes/KOH, pH 7.4, 1 mM-EDTA, 0.05% (w/v) defatted BSA and 8.5% (w/v) PEG. The mitochondria were diluted 5-fold (final protein concn. 20 mg/ml) with ice-cold Medium X in a 20 ml glass scintillation vial, and toluene was added to a final concentration of 0.2% (v/v); the vial was immediately capped and gently agitated by hand in an ice bath for 3 min, with occasional inversion. The mixture was quickly diluted with 15 vol. of ice-cold Medium X, and immediately centrifuged at 3000 ***g*** for 5 min at 4 °C in an SS-34 rotor in a Sorvall RC-5 centrifuge. The permeabilized mitochondria were resuspended gently in Medium X, in a volume twice that of the permeabilization mixture, sedimented at 1500 ***g*** for 3 min as above, and resuspended in Medium X to a final protein concentration of 25–35 mg/ml. Protein in the permeabilized mitochondrial preparations was measured by the method of Lowry *et al.* [22], with BSA as the standard.

### Incubation of permeabilized mitochondria

All incubations were done in duplicate, in air, in capped 7 ml plastic vials, at 25 °C in a Dubnoff shaking bath. The standard incubation mixture contained (final concns.) 50 mM-Tris/HCl, pH 7.4, 2 mM-EDTA, 7 mM-$MgCl_2$, 0.1 M-mannitol, 10 mM-ornithine, 0.1% defatted BSA and 8.5% PEG. For some studies of OCT, the standard medium also contained carbamoyl phosphate at various concentrations, given in the Results section. For studying the synthesis of carbamoyl phosphate and citrulline *de novo*, the standard medium described above was supplemented with 5 mM-$KH_2PO_4$, pH 7.4, 10 mM-$NH_4Cl$, 15 mM-$KHCO_3$, 5 mM-ATP, 5 mM-*N*-acetylglutamate, 10 mM-succinate, 30 $\mu$M-atractylate, 10 $\mu$g of oligomycin (including 0.2% ethanol)/ml, 2.5 units of creatine kinase/ml and 5 mM-phosphocreatine. In some experiments [$^{14}$C]$HCO_3^-$ was used, at 2–8 $\mu$Ci/ml. The final pH was 7.4, the final volume was 1 ml, and the mitochondrial protein concentration was 2.5–3.5 mg/ml. The pH of the incubation medium did not change during the short incubations used in this work. The reactions were generally started by the addition of mitochondria to prewarmed incubation mixtures. The concentrations of the substrates listed above were varied as required for determining various kinetic properties of the enzymes; all variations are described in the Results section.

Since some CPS and OCT were present in the medium, the total activities of these enzymes measured in the incubations represent the sum of the activities present in mitochondria and medium. In order to know the mitochondrial activities, the activities in the medium were measured as follows: mitochondria were separated from incubation mixtures by centrifugation through 0.4 ml of silicone 550 oil (see below) in 1.5 ml microcentrifuge tubes; samples of the supernatant were removed and incubated as required. All activities shown as occurring in mitochondria have been corrected for enzyme activity in the medium.

For the determination of citrulline, the reactions were stopped by addition of $HClO_4$ (final concn. 1.7 M); when carbamoyl phosphate was to be measured, further CPS activity was stopped and preformed carbamoyl phosphate was converted into citrulline as previously described [1,2], or into urea as described in [23]. Citrulline was measured in the protein-free supernatants either colorimetrically [1], or where applicable, by liquid-scintillation counting after heating the samples at 70 °C for 45 min to drive off unchanged $H^{14}CO_3^-$. Myokinase activity in mitochondrial preparations was measured as described in [24], and rotenone-insensitive NADH–cytochrome *c* reductase as in [25], with 5 $\mu$M-rotenone. Total activities of OCT and CPS were assayed as in [26] and [27] respectively, in intact and permeabilized mitochondria to determine recoveries of these enzymes in the latter.

For incubations designed to determine the water-, sucrose- or citrulline-permeable spaces of mitochondria, the standard incubation mixture described above also contained $^3H_2O$ (2 $\mu$Ci/ml) plus [$^{14}$C]sucrose (1.25 $\mu$Ci/ml) or [*ureido*-$^{14}$C]citrulline (1.25 $\mu$Ci/ml). The mitochondrial spaces permeable to these tracers were determined by the centrifugal filtration method as described previously [28], except that undiluted 550 silicone oil was used instead of the usual mixture of 550 and 200 oils [12]; this modification was necessary because of the high density of the PEG-containing incubation mixtures. The quenching layer under the oil was generally 14% (w/v) $HClO_4$. The permeability of [$^{14}$C]carbamoyl phosphate (0.1–0.5 $\mu$Ci/ml of incubation) was determined in a similar fashion, except that the quenching solution consisted of an ice-cold solution of 7% $HClO_4$, 0.5 M-mannitol and 20 mM-EDTA [17]. Immediately after centrifugation, portions of each supernatant solution were placed in 7 ml vials, which were covered at once with rubber stoppers fitted with centre wells containing 0.15 ml of a solution of ethylene glycol/ethanolamine (2:1, v/v); 0.5 ml of 14% $HClO_4$ was then injected into each vial. The remainder of each supernatant and most of the oil were removed from the centrifugation tubes, and 80 $\mu$l of the underlying solution was removed from each tube with a Hamilton syringe, placed in vials, and treated in the same way as the supernatants. The vials were left overnight to allow complete absorption of evolved $CO_2$ by the solution in the centre well. The wells were then removed and placed in scintillation vials, and 0.15 ml of water and 4 ml of Hydrofluor were added for liquid-scintillation counting.

### Immobilization of enzymes

Activated CH-Sepharose 4B was swollen and washed several times with 1 mM-HCl, and then washed once with coupling buffer (0.1 M-$KHCO_3$/0.5 mM-dithiothreitol, pH 8.0). Purified CPS, which was stored at −75 °C in 0.3 M-$KH_2PO_4$/5 mM-ornithine/0.5 mM-dithiothreitol, was passed through Sephadex G-25 (equilibrated with coupling buffer) immediately before immobilization. When OCT was to be co-immobilized, purified OCT in 1.7 M-$(NH_4)_2SO_4$ was added to the CPS solution after the Sephadex G-25 step. Defatted BSA was added to a final concentration of 0.1% (w/v), and the enzyme solution was mixed end-over-end with the gel (100 mg of original dry weight

of gel/ml) for 2 h at 4 °C. The gel was then mixed for 1 h with 0.1 M-Tris/HCl/0.5 M-KCl/0.5 mM-dithiothreitol, pH 8.0, washed three times with the Tris/KCl/dithiothreitol solution, and once with coupling buffer, and finally resuspended in coupling buffer.

Although several concentrations of enzymes and substrates were examined for their effects on the immobilization of the enzymes, the conditions generally used were as follows: CPS, 0.6–0.8 unit/ml of coupling reaction; OCT, 16 units (measured at pH 7.4; see below)/ml; $NH_4^+$ [from the $(NH_4)_2SO_4$ of the OCT preparation], 25 mM; defatted BSA, 0.06%. The activity of CPS was assayed in a shaking bath at 37 °C in a total volume of 0.5 ml at pH 7.4 in a medium containing 50 mM-Hepes/KOH, 50 mM-$NH_4HCO_3$, 9 mM-$MgSO_4$, 5 mM-*N*-acetylglutamate, 2 mM-EDTA, 0.2% defatted BSA, 5 units of creatine kinase, 5 mM-phosphocreatine, 5 mM-ATP and 5 mM-ornithine. For assays of soluble CPS the mixture also included 0.4 unit of OCT. The samples were preincubated in the incubation mixtures for 5 min without ATP, and the reactions were started by addition of ATP. After 10 min, CPS activity in parallel incubations was stopped by addition of either $HClO_4$ (final concn. 1.7 M) or EDTA (final concn. 25 mM). In the latter case the incubations were continued for 10 min so that any existing carbamoyl phosphate would be converted into citrulline, and then they were stopped with $HClO_4$. The activity of co-immobilized OCT and of soluble OCT in the enzyme mixtures used for immobilization was measured in the same medium, except that ATP and the ATP-regenerating system were omitted, and the reactions were started by addition of carbamoyl phosphate (final concn. 5 mM). The affinities of carbamoyl phosphate and ornithine for OCT were determined both in the mixture described above and in a medium containing 200 mM-triethanolamine, pH 8.5; the fixed substrate was at 10 mM, and the variable one 0.05–10 mM.

## RESULTS

### Characteristics of the permeabilized mitochondria

The permeabilized mitochondria retained 89% of the OCT and CPS activity of the original mitochondrial preparations (Table 1). Small amounts of both OCT and CPS were found in the medium during incubations of both intact and permeabilized mitochondria under the standard conditions used, i.e. 8.5% PEG present (Table 1). (At lower PEG concentrations, e.g. 2 or 4%, higher percentages of the enzymes were found in the incubation medium of permeabilized mitochondria; similar results were reported for citrate synthase by Matlib *et al.* [15].) The values shown in Table 1 are for incubations from 15 s to 1 min for OCT or 5 min for CPS. The activities of rotenone-insensitive NADH–cytochrome *c* reductase, an enzyme of the outer membrane, and of myokinase, an enzyme of the intermembrane space, were nearly the same in the intact and permeabilized mitochondria (results not shown), indicating that the outer membrane was retained in the latter.

**Table 1. Characteristics of permeabilized mitochondria**

The methods used to prepare untreated and permeabilized mitochondria and to determine enzyme activities and water- and sucrose-permeable volumes are described in the Materials and methods section. The values shown are means ± S.D., with the numbers of experiments given in parentheses.

| | Untreated mitochondria | Permeabilized mitochondria |
|---|---|---|
| OCT and CPS activity retained (%) | 100 | 89 ± 13 (4) |
| OCT activity in medium during incubations (%) | 3 ± 0.3 (5) | 9 ± 4 (6) |
| CPS activity in medium during incubations (%) | < 3 | 4 |
| Ratio of $^3H_2O$/[$^{14}C$]sucrose volumes | 1.6 ± 0.3 (6) | 1.1 ± 0.1 (33) |

Comparison of the ratios of the $^3H_2O$- and [$^{14}C$]sucrose-permeable volumes in intact and permeabilized mitochondria shows that the matrix space of the treated mitochondria was accessible to sucrose (Table 1). Similar results (not shown) were obtained with [$^{14}C$]citrulline. The ratio of the $^3H_2O$ and [$^{14}C$]sucrose volumes was measured in every experiment.

Since sucrose was freely permeant, it was not possible to determine the water volume of the matrix compartment by the usual method, which relies on the difference between the $^3H_2O$- and the [$^{14}C$]sucrose-permeable volumes. Attempts to use radioactively labelled inulin ($M_r$ 5000) or dextran ($M_r$ 70000) to distinguish between the matrix and intermembrane or adherent water spaces, respectively, were unsuccessful because these compounds behaved anomalously: the water/inulin ratios were always less than 1, and the dextran volumes were affected by [PEG] non-linearly.

The total $^3H_2O$ volume ($\mu$l/mg of protein) of the permeabilized mitochondria was $2.14 \pm 0.23$ ($n = 19$), and that of intact mitochondria in the same incubation medium was $2.21 \pm 0.16$ ($n = 4$). Assuming that 30–40% of the total water space was adherent water [28] and given that permeabilized mitochondria have an intermembrane space (see above), the matrix space of permeabilized mitochondria appeared to be similar to that of intact mitochondria in iso-osmotic medium [28].

### Kinetic properties of OCT in permeabilized mitochondria

When permeabilized mitochondria were incubated in the standard medium with 10 mM-ornithine and 5 mM added carbamoyl phosphate, citrulline was formed by OCT, both in the mitochondria and in the medium. In four experiments 425 ± 211 (mean ± S.D.) nmol of citrulline/min per mg of protein was made in the matrix. In a representative experiment, 400 nmol of citrulline was formed by 2.5 mg of mitochondrial protein in 30 s, 280 nmol in the mitochondria and 120 nmol in the medium. Since, at most, 10% of the total OCT is found in the medium (see above), these data indicate that the activity of the enzyme remaining in the matrix is greatly restricted. The mitochondria were freely permeable to added carbamoyl phosphate; after incubation for 10–15 s with 0.2 mM-, 2 mM- or 5 mM-[$^{14}C$]-carbamoyl phosphate (no ornithine present), essentially the same respective concentrations of carbamoyl phosphate were measured in the total pellet water.

The kinetic properties of OCT in the permeabilized mitochondria were markedly different from those observed for the soluble enzyme in the same medium, the apparent $K_m$ values being higher for OCT *in situ* (Table 2). The most striking difference was for carbamoyl phosphate; its apparent $K_m$ for OCT in the matrix was about 8 times that for the enzyme in solution. The incubation media used in the experiments in Table 2 inhibit OCT in solution by about 50%, but the apparent $K_m$ values of both substrates for the soluble enzyme are similar to those measured under optimal assay conditions [29,30].

OCT activity in the matrix and in the medium was inhibited still further in the presence of 5 mM-$KH_2PO_4$, and the $s_{0.5}$ for carbamoyl phosphate appeared to be higher. For example, in a typical experiment, OCT in the matrix formed 422 and 680 nmol

**Table 2. Kinetic properties of OCT in permeabilized mitochondria**

For measurement of the apparent $K_m$ for carbamoyl phosphate, permeabilized mitochondria were incubated in the standard medium, as described in the Materials and methods section, except that [ornithine] was 5 mM, and 0.1–5 mM-[$^{14}$C]carbamoyl phosphate was used (0.04–0.8 $\mu$Ci/ml of incubation). For measurement of the apparent $K_m$ for ornithine, permeabilized mitochondria were incubated in the mixture used to support endogenous carbamoyl phosphate synthesis, except that $KH_2PO_4$ was omitted and 10 mM-carbamoyl phosphate was added, with ornithine from 0.2 to 7.5 mM. The mitochondrial protein concentration was 0.2–0.5 mg/ml. The properties of OCT in the medium were determined after removing the permeabilized mitochondria from the medium by centrifugal filtration as described in the Materials and methods section. Incubations were for 15 s. The values shown are means ± S.D.; $n = 4$ for variable carbamoyl phosphate, $n = 3$ for variable ornithine.

| | Apparent $K_m$ for | |
|---|---|---|
| | Carbamoyl phosphate (mM) | Ornithine (mM) |
| OCT in mitochondrial matrix | 0.40 ± 0.04 | 0.98 ± 0.28 |
| OCT in medium | 0.051 ± 0.013 | 0.51 ± 0.11 |

**Table 3. Effects of PALO on OCT in permeabilized mitochondria**

Permeabilized mitochondria were preincubated with PALO for 5 min in the standard medium, supplemented to support carbamoyl phosphate synthesis *de novo*, as described in the Materials and methods section, except that $NH_4Cl$ was omitted. The reactions were started with either carbamoyl phosphate (final concn. 5 mM), or (for endogenous carbamoyl phosphate synthesis) with $NH_4Cl$ (final concn. 10 mM). The incubations were for 30 s–1 min. Abbreviation: CAP, carbamoyl phosphate.

| | | Inhibition by PALO (%) | | |
|---|---|---|---|---|
| | | OCT in medium | OCT in matrix | |
| | [PALO] | Added CAP (1) | Endogenous CAP (2) | Added CAP (3) |
| Expt. 1 | 1 $\mu$M | 86 | 22 | 31 |
| Expt. 2 | 2.5 $\mu$M | 95 | 63 | 87 |
| Expt. 3 | 0.1 mM | 95 | 92 | 89 |

of citrulline in 30 s with 1 mM and 5 mM added carbamoyl phosphate, respectively, in the standard medium, but only 156 and 406 nmol in the complete incubation medium used to support CPS activity, which contained 5 mM-$KH_2PO_4$. OCT in the medium, i.e. soluble OCT, formed 85 and 104 nmol in the standard medium and 56 and 136 nmol in the phosphate-containing medium used for CPS activity, at 1 mM and 5 mM added carbamoyl phosphate respectively.

$P_i$ is a competitive inhibitor of rat liver OCT, with a $K_i$ of 0.25 mM for the purified rat enzyme at pH 8.1 [29,30]; the mouse liver enzyme is similarly inhibited by $P_i$ [27]. The number of experimental conditions that could be used in a given experiment was insufficient to allow a precise determination of the $K_i$ for $P_i$ in permeabilized mitochondria. Assuming competitive inhibition, an approximate $K_i$ of 1–2 mM at pH 7.4 was calculated.

The transition-state analogue PALO is an inhibitor of OCT, competitive with carbamoyl phosphate, with a $K_i$ of 0.25 $\mu$M [31,32]. At 1 $\mu$M and 2.5 $\mu$M, PALO inhibited OCT in the incubation medium by 86 and 95 %, respectively, in the presence of 5 mM-carbamoyl phosphate (Table 3, column 1). This inhibition is greater than that calculated from the $K_i$ value, and may be related to the composition of the medium. At each of those concentrations PALO was much less inhibitory for matrix OCT when carbamoyl phosphate was generated endogenously and simultaneously by matrix CPS (Table 3, column 2), even though the levels of carbamoyl phosphate in the matrix under the latter conditions should have approached 3 nmol/$\mu$l (see below); the matrix content of carbamoyl phosphate was essentially the same in the presence and absence of PALO (results not shown). PALO was also less inhibitory for matrix OCT than for OCT in the medium when 5 mM added carbamoyl phosphate was used (Table 3, column 3). At high (0.1 mM) PALO concentrations, however, matrix OCT was inhibited to the same extent with carbamoyl phosphate from either source, and similarly to soluble OCT (Table 3).

## Characteristics of citrulline synthesis *de novo* by permeabilized mitochondria

The permeabilized mitochondria synthesized carbamoyl phosphate from ammonia, bicarbonate and ATPMg, in the presence of *N*-acetylglutamate. The addition of both ATPMg and acetylglutamate was obligatory. In 5 min incubations under these conditions, carbamoyl phosphate was formed at a velocity of 22 ± 3 nmol/min per mg (mean ± S.D., $n = 3$). When ornithine was also present, about 80 % of the carbamoyl phosphate was converted into citrulline (Table 4), mostly in the matrix. [We have alrady shown that only a small portion of endogenous carbamoyl phosphate escapes into the medium; this is inferred from the fact that PALO had little effect on citrulline synthesis from endogenous carbamoyl phosphate, whereas it greatly inhibited synthesis by soluble OCT (Table 3).]

In contrast with the behaviour of intact mitochondria, in which CPS becomes severely inhibited in the absence of ornithine [1], the velocity of carbamoyl phosphate synthesis by permeabilized mitochondria was the same in the presence or absence of ornithine (Table 4). Reflecting the fact that the mitochondria were permeabilized, and that carbamoyl phosphate and citrulline should therefore have diffused out of the matrix quickly, the levels of these compounds in the mitochondria were similar to those in the medium, with one important exception: in the presence of ornithine, that is, when citrulline was being formed, mitochondrial levels of carbamoyl phosphate were 1.0–1.5 nmol/$\mu$l of total pellet water, many times higher than the concentration of carbamoyl phosphate in the medium (Table 4). The levels of carbamoyl phosphate in the matrix were undoubtedly even higher than 1.0–1.5 nmol/$\mu$l, since a considerable portion (at least half) of the total pellet water must be adherent and intermembrane-space water [28]. It is unclear how such a difference is maintained; the exit of endogenously formed carbamoyl phosphate is restricted, although the mitochondria are freely permeable to added carbamoyl phosphate (see above).

## Preferential use of endogenous carbamoyl phosphate by matrix OCT

The observations described above suggested that OCT in the matrix may use endogenously synthesized carbamoyl phosphate preferentially, that is, even in the presence of relatively high concentrations of exogenous carbamoyl phosphate. To test this, [$^{14}$C]$HCO_3^-$ was used as a substrate, and the extent of labelling of carbamoyl phosphate and of citrulline was determined in the

**Table 4. Synthesis *de novo* of carbamoyl phosphate and citrulline in permeabilized mitochondria; mitochondrial levels of carbamoyl phosphate and citrulline**

Permeabilized mitochondria were incubated for 5 min in the standard medium supplemented to support the synthesis *de novo* of carbamoyl phosphate, with or without ornithine, as described in the Materials and methods section. The results of three separate experiments are shown. To measure mitochondrial levels of the intermediates of interest, the incubations were performed with $[^{14}C]HCO_3^-$, and the mitochondria were separated from the medium by centrifugation through silicone oil into 0.1 ml of either 14% $HClO_4$ when citrulline was to be measured, or a solution of 19% (w/v) mannitol, 1% (v/v), Triton X-100, 25 mM-EDTA and 50 mM-Tris, pH 7.4, for measurement of carbamoyl phosphate. In Expts. 1 and 2, the latter was measured in the mitochondrial extracts after conversion into citrulline by addition of 10 mM-ornithine, 12 units of bovine OCT and additional Tris to maintain its concentration and the pH. The mixtures were incubated for 10 min at 25 °C, and the reactions were stopped by acidification (1.7 M-$HClO_4$, final concn.). In Expt. 3 carbamoyl phosphate was measured after conversion into urea by boiling for 10 min in 0.6 M-$(NH_4)_2SO_4$ [23]. The mitochondrial protein concentration used was 3.1, 3.2 and 3.2 mg/ml of incubation in the three experiments. All data shown for synthesis represent that occurring inside the mitochondria, i.e. they have been corrected for synthesis occurring in the medium. Abbreviation: CAP, carbamoyl phosphate. Mitochondrial contents are expressed as nmol of intermediate/$\mu$l of total pellet water.

| | | Amounts present (nmol) as: | | Mitochondrial content (nmol/$\mu$l) | |
|---|---|---|---|---|---|
| Expt. | Ornithine added | CAP | Citrulline | CAP | Citrulline |
| 1 | None | 385 | 0 | 0.35 | 0 |
| | 10 mM | 61 | 337 | 1.53 | 0.60 |
| 2 | None | 312 | 7 | 0.20 | 0 |
| | 10 mM | 78 | 263 | 1.01 | 0.60 |
| 3 | None | 287 | 0 | 0.23 | 0 |
| | 10 mM | 30 | 257 | 1.48 | 0.22 |

absence and presence of added excess unlabelled carbamoyl phosphate. In each of three experiments, addition of 5 mM unlabelled carbamoyl phosphate resulted in a decrease of about 50% in the amount of labelled citrulline formed; most of this decrease, however, is attributable to inhibition of CPS activity by the added carbamoyl phosphate, consistent with the measured $K_i$ for this compound (see below). The conversion of labelled endogenous carbamoyl phosphate into citrulline was essentially unaffected by the addition of unlabelled carbamoyl phosphate, as shown in detail for a representative experiment in Table 5. In the absence of added carbamoyl phosphate, 56% of the total carbamoyl phosphate formed was converted into citrulline; this percentage was decreased only slightly, to 49% and 46%, by addition of 1 mM and 5 mM unlabelled carbamoyl phosphate respectively. Addition of 5 $\mu$mol of unlabelled carbamoyl phosphate (5 mM) represents a dilution of total endogenous labelled carbamoyl phosphate of approx. 200-fold.

It was ascertained (Table 5) first, that some of the exogenous carbamoyl phosphate was utilized by OCT in the matrix; second, that a substantial amount of the added carbamoyl phosphate remained at the end of the incubation, so the results described above were not merely due to utilization of all of the added carbamoyl phosphate; third, that the percentage of added carbamoyl phosphate used was only 20% and 10% at 1 mM- and 5 mM-carbamoyl phosphate respectively, whereas almost 50% of the endogenously generated intermediate was converted into citrulline both in the absence and in the presence of added carbamoyl phosphate (see above, and Table 5). In contrast, when lysed mitochondria were used, all of the newly formed carbamoyl phosphate was converted into citrulline if no carbamoyl phosphate was added, but only 74% and 24% was converted in the presence of 1 mM and 5 mM added carbamoyl phosphate respectively.

All of these data are consistent with the conclusion that endogenously generated carbamoyl phosphate has preferential access to matrix OCT or to a portion of it. These findings agree with those of Wanders *et al.* [17], which indicated partial channelling of endogenous carbamoyl phosphate to OCT. Those authors, however, also found evidence suggestive that newly formed and existing carbamoyl phosphate equilibrate sufficiently rapidly as to constitute a single pool; on this basis, they considered that the partial channelling of carbamoyl phosphate could be ignored in calculating the elasticity coefficient of matrix OCT for this substrate [17].

**Kinetic properties of CPS in permeabilized mitochondria**

Under optimal conditions the activation of CPS by *N*-acetylglutamate is essentially instantaneous [33]. As previously reported [16], the activation of CPS by acetylglutamate in permeabilized mitochondria was extremely slow, especially at low concentrations of the latter and in the absence of ATPMg. We established that this is also true of CPS in solution in the standard incubation medium used in these experiments. For example, at 0.1 mM- and 0.2 mM-acetylglutamate, with 5 mM-ATPMg, CPS activity in solution increased for at least 20 min, which was the longest preincubation time we studied. At 5 mM-acetylglutamate, with ATPMg present, activation occurred in less than 5 min. The slowness of activation may be related to the composition of the standard incubation medium, which is greatly inhibitory for CPS, especially at low [acetylglutamate]. For example, after 5 min preincubation with acetylglutamate and 5 mM-ATPMg, followed by a 5 min incubation, CPS was inhibited by 80 and 40% when acetylglutamate was 0.1 mM and 5 mM respectively, compared with the activity obtained in optimal medium. PEG, mannitol, atractylate, oligomycin or succinate were not individually responsible for the inhibition. The medium generally used for incubation of isolated intact mitochondria in studies of citrulline synthesis also inhibits soluble CPS profoundly [34].

To determine the apparent $K_m$ for $NH_3$ [20], 0.1–5 mM-$NH_4Cl$ was used; incubations were for 30 s–1 min. In permeabilized mitochondria the value obtained in three experiments was 3–4 $\mu$M, and it was the same for soluble CPS in the standard incubation medium used in this work. The same results were obtained whether or not permeabilized mitochondria or soluble CPS were preincubated for 5 min with acetylglutamate and ATPMg (5 mM each). We determined previously [20] that the apparent $K_m$ of $NH_3$ for CPS in intact isolated rat liver mitochondria is about 3-fold lower than for the soluble purified rat enzyme in optimal medium. The apparent $K_m$ of ATPMg for

**Table 5. Preferential use of endogenously formed carbamoyl phosphate by matrix OCT**

Permeabilized mitochondria were incubated with [$^{14}$C]$HCO_3^-$ in the absence and presence of added unlabelled carbamoyl phosphate (CAP) as described in the text. The values shown are total nmol formed in 30 s in a 1 ml incubation in a representative experiment. Of the total citrulline synthesized in the presence of added carbamoyl phosphate (lines 2 and 3), 80 and 264 nmol were formed by OCT in the matrix when the concentration of added carbamoyl phosphate was 1 mM and 5 mM respectively. Abbreviations: [$^{14}$C]Cit, citrulline synthesized from endogenously formed carbamoyl phosphate; [$^{14}$C]CAP, endogenously formed carbamoyl phosphate remaining as such at the end of the incubation.

| Addition | [$^{14}$C]Cit (nmol) | [$^{14}$C]CAP (nmol) | Total citrulline (nmol) |
|---|---|---|---|
| None | 14.5 | 11.2 | 14.5 |
| 1 mM-CAP | 10.8 | 11.3 | 186 |
| 5 mM-CAP | 6.9 | 8.2 | 465 |

CPS, whether in permeabilized mitochondria or soluble, was 0.71 mM, and the concentration of added carbamoyl phosphate required for 50% inhibition of CPS at 1 mM-ATPMg was 10–15 mM. These values are similar to those reported by Lof *et al.* [16], using permeabilized mitochondria, and to those reported for purified CPS [35,36].

**Behaviour of co-immobilized CPS and OCT**

The best recoveries of CPS activity after immobilization, 11–20%, were obtained in the presence of $NH_4^+$ or ethanolamine plus BSA; in the absence of $NH_4^+$ recoveries were only about 5%. All of the CPS added was bound; it could not be determined whether the loss of activity reflected inactivation of CPS during binding, or a lower molecular activity of CPS whose mobility might be restricted by binding. In one experiment, the presence of 50 mM-carbamoyl phosphate during coupling increased CPS recovery to 24%.

The recovery of OCT activity after immobilization was consistently slightly greater than 100%. The apparent $K_m$ values of carbamoyl phosphate and ornithine for immobilized OCT were 2–4 mM and 4–5 mM respectively; these are about 10 times those for soluble OCT measured under the same conditions.

When the co-immobilized enzymes were incubated with ornithine and the substrates of CPS, essentially all of the carbamoyl phosphate formed was converted into citrulline; in a typical experiment about 30 nmol of citrulline was formed/10 min per 0.5 ml of incubation mixture. When ornithine was omitted, the carbamoyl phosphate formed was released into the medium, reaching approx. 60 $\mu$M concentration. If ornithine was then added (with EDTA to stop CPS activity), the carbamoyl phosphate was used by OCT at a velocity only 68% of that observed when all substrates were added simultaneously; this is consistent with the high apparent $K_m$ of immobilized OCT for added carbamoyl phosphate mentioned above. The markedly different utilization of carbamoyl phosphate under these two conditions suggests either that locally high concentrations of carbamoyl phosphate are reached near the catalytic site of OCT when CPS is active, or that the high apparent $K_m$ for carbamoyl phosphate present in the medium is strictly a function of the limited accessibility of the catalytic site of the immobilized OCT. These characteristics of co-immobilized OCT resemble those of OCT in intact mitochondria with respect to the utilization of endogenously generated compared with exogenous carbamoyl phosphate [2], and also those of OCT in permeabilized mitochondria, described above and shown in Table 5. The inhibition of CPS in the absence of ornithine which is characteristic of intact mitochondria could not be reproduced with the co-immobilized enzymes.

## DISCUSSION

The permeabilized mitochondria used in these studies retained most of the CPS and OCT of the original mitochondria, and were stable during incubations in the presence of 8.5% PEG, as noted by Matlib *et al.* [15]. In our hands, permeabilization with 2% toluene [15] resulted in lysis of the mitochondria, whereas treatment with 0.2% toluene for 3 min as described by Lof *et al.* [16] gave satisfactory and reproducible results, and was therefore used routinely. The matrix volume of the treated mitochondria in the standard incubation medium was similar to that measured in intact mitochondria in iso-osmotic media, i.e. the permeabilized mitochondria were not swollen. The activities of enzymes located in the outer membrane and the inter-membrane space were similar in intact and permeabilized mitochondria.

In previous work we showed that CPS and OCT in intact isolated mitochondria do not function as might be expected if they were simply soluble enzymes homogeneously distributed in the matrix. For example, mitochondrial CPS is severely inhibited in the absence of ornithine, by a mechanism that seemingly does not involve product inhibition [1,2]. OCT uses extramitochondrial ornithine in preference to matrix ornithine [3]; in contrast, it does not readily use added carbamoyl phosphate, and is poorly inhibited by PALO, even though these compounds permeate and reach matrix concentrations which would be saturating for soluble OCT [2].

In this work we show that some of these special properties of OCT are also demonstrable in permeabilized mitochondria, in which membrane barriers to low-molecular-mass compounds do not exist. The apparent $K_m$ values for added carbamoyl phosphate and ornithine are respectively 8 and 2 times those of OCT in solution, and PALO is much less inhibitory, especially when carbamoyl phosphate is synthesized endogenously rather than added at 5 mM, or about 10 times the apparent $K_m$ for matrix OCT (Table 2). These findings suggest that the binding of these added compounds to OCT is hindered in mitochondria. We do not know whether the binding sites are less accessible to the added compounds, or whether the conformation of OCT is such that its affinity for carbamoyl phosphate and PALO is less than in solution. The fact that the incorporation of endogenously synthesized [$^{14}$C]carbamoyl phophate into citrulline is little affected by a 200-fold excess of unlabelled added carbamoyl phosphate favours the first possibility.

The effects that restrictions to mobility can have on the catalytic properties of OCT are exemplified by observations made with preparations of co-immobilized CPS and OCT. We found that the apparent $K_m$ of OCT for added carbamoyl phosphate and ornithine was 10 times that of the soluble enzyme, and that OCT used carbamoyl phosphate concurrently synthesized by CPS better than it did carbamoyl phosphate in the medium.

In the incubation medium used in these studies, the activation by acetylglutamate of CPS in permeabilized mitochondria ([16]; the present work) or in solution (the present work) is anomalously slow. The properties of CPS with respect to acetylglutamate may differ in the matrix of intact mitochondria and in solution; we previously reported that added acetylglutamate rapidly activates this enzyme in uncoupled, but not in coupled, intact isolated

mitochondria, even though it permeates equally well into both [37].

The effect of ornithine on CPS of intact mitochondria [1] was not detected in permeabilized mitochondria; it is possible that it requires the participation of the inner membrane and the ornithine transporter in ways which are disrupted by permeabilization with toluene. A finding that we cannot explain is how an apparently large gradient of newly synthesized carbamoyl phosphate is maintained between permeabilized mitochondria and medium during citrulline synthesis *de novo*. The exit of carbamoyl phosphate from permeabilized mitochondria appears to be as hindered as the access of added carbamoyl phosphate is to mitochondrial OCT. There appeared to be no permeability barrier to the entry of carbamoyl phosphate in these preparations, as determined by direct measurement. Consistent with this, addition of 5 mM-carbamoyl phosphate resulted in 50% inhibition of matrix CPS. Yet the added compound did not interfere with the conversion of endogenously formed carbamoyl phosphate into citrulline.

Evidence obtained in intact mitochondria has been interpreted to mean that some kinetic properties of some enzymes are different *in situ* and in solution in metabolically important ways [1–3,37,38]. It is crucial in these cases to ascertain that such evidence is not an artifact arising from permeability barriers [1–3,37,39]. This is not so when permeabilized mitochondria are used. Hence, kinetic differences between enzymes in solution and in permeabilized mitochondria ([40–42]; the present work) must be related to the inherent properties of the inner membrane and the matrix components which are retained in these preparations. Since the composition of incubation media is vastly different from that of the matrix, and must alter the latter, it could be argued that this alone is responsible for the observed differences. We have eliminated this possibility for OCT by determining that the properties of matrix OCT differ from those of the soluble enzyme in the same medium.

Our observations on the effects of PALO on OCT in permeabilized mitochondria are similar to those made by Wanders *et al.* [17] using norvaline as the inhibitor of OCT, and to those we made previously using PALO and intact mitochondria [2]: in all these cases, matrix OCT was less inhibited when carbamoyl phosphate was generated in the matrix than when it was added to the medium. Wanders *et al.* [17] postulated that the effect of the inhibitor on citrulline synthesis is related to the low flux control coefficient of matrix OCT under the conditions used.

At present, however, calculations of flux control and elasticity coefficients of matrix enzymes such as OCT and CPS are plagued by many numerical and theoretical difficulties. The volume of free, osmotically active, matrix water is not known with certainty [5,43,44]; under iso-osmolar conditions it is only 50–60% of the total matrix water [43,44]. Consequently, the molarity of reactants in the matrix and all quantities calculated from these, such as matrix pH, cannot be firmly established, and flux control and elasticity coefficients cannot be accurately calculated. Moreover, the bulk matrix concentration of a substrate may not be the relevant one. The velocity of OCT is a function of extramitochondrial ornithine channelled to it, not of the matrix [ornithine] [3]. Similarly, carbamoyl phosphate is partially channelled to OCT ([17]; the present work).

We propose that the unique behaviour of matrix OCT, such as the preferential utilization of endogenous rather than exogenous carbamoyl phosphate, the higher $K_m$ value for ornithine, and the lower apparent affinity for PALO, may reflect kinetic properties of OCT which are only evident in its native macromolecular environment, or steric constraints on the access of reactants to sites on the enzyme. These are working hypotheses to be tested in future studies.

We have previously shown that, in intact mitochondria, extramitochondrial ornithine is channelled to matrix OCT during citrulline synthesis [3], and that in isolated permeabilized hepatocytes other endogenously generated urea-cycle intermediates are preferentially used by each of the three cytoplasmic enzymes of this pathway [45]. The studies described here suggest that carbamoyl phosphate is channelled between CPS and OCT in permeabilized mitochondria (see also [17]). The entire pathway of urea synthesis thus appears to be organized. Such functional organization of soluble enzymes may exist in many metabolic pathways [9].

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