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Altered enzyme activities and citrulline synthesis in liver mitochondria from ornithine carbamoyltransferase-deficient *sparse-fur*^{ash} mice

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Male mice carrying the *spf*^{ash} mutation have 5–10 % of the normal activity of ornithine carbamoyltransferase, yet are only slightly hyperammonaemic and develop quite well. A study of liver mitochondria from normal and *spf*^{ash} males showed that they differ in important ways. (1) The *spf*^{ash} liver contains about 33 % more mitochondrial protein per g than does normal liver. (2) The specific activities of carbamoyl-phosphate synthetase (ammonia) and glutamate dehydrogenase are about 15 % lower than normal in mitochondria from *spf*^{ash} mice, whereas those of β-hydroxybutyrate dehydrogenase and cytochrome oxidase are 22 % higher and 30 % lower respectively. (3) In the presence of 10 mM-ornithine and the substrates for carbamoyl phosphate synthesis, coupled and uncoupled mitochondria from *spf*^{ash} mice synthesize citrulline at unexpectedly high rates, about 25 and 44 nmol/min per mg respectively. Though these are somewhat lower than the corresponding rates obtained with normal mitochondria, the difference does not arise from the deficiency in ornithine carbamoyltransferase, but from the lower carbamoyl-phosphate synthetase activity of the mutant mitochondria. (4) At lower external [ornithine] (< 2 mM), a smaller fraction of the carbamoyl phosphate synthesized is converted into citrulline in *spf*^{ash} than in normal mitochondria. These studies show that what appears to be a single mutation brings about major adaptations in the mitochondrial component of liver. In addition, they clarify the role of ornithine transport and of protein–protein interactions in citrulline synthesis in normal mitochondria.

INTRODUCTION

Ornithine carbamoyltransferase (OCT; EC 2.1.3.3) catalyses the formation of citrulline from carbamoyl phosphate and ornithine. With carbamoyl-phosphate synthetase (ammonia) (CPS; EC 6.3.4.16), it forms the mitochondrial portion of the urea cycle. These enzymes are found only in liver cells and in enterocytes [1,2]; they are located exclusively in the mitochondrial matrix [3,4], and under normal conditions behave as a coupled system [5,6].

Both CPS and OCT are nuclear-coded proteins [7,8], the OCT gene being on the X chromosome [9]. Two different OCT-deficient mouse mutants have been described: in sparse-fur (*spf*), a K_m mutant, the livers of the affected males have enzyme protein contents higher than those found in normal mice [9,10]; in sparse-fur-abnormal skin and hair (*spf*^{ash}) [11], the OCT appears to be identical with the normal enzyme, but the livers of these males contain only 5–10 % of normal amounts of the protein [10,12].

The *spf*^{ash} males have only mild hyperammonaemia and survive well on a normal diet, whereas humans with similarly profound OCT deficiency are grossly hyperammonaemic and often do not survive when kept on a normal diet [13,14]. It is puzzling that the *spf*^{ash} mouse can function so well with so little OCT; consequently we have examined the capacity of isolated *spf*^{ash} mitochondria for citrulline synthesis, and we describe here some characteristics of the behaviour of *spf*^{ash} OCT *in situ*.

We have found that the livers of *spf*^{ash} mutants contain 33 % more mitochondrial protein per g than do those of normal mice. We also describe major changes in the total activities of other mitochondrial enzymes in *spf*^{ash} liver, and in the specific activities of those enzymes in the mitochondria.

MATERIALS AND METHODS

Animals and reagents

The *spf*^{ash} mice were generously given by Dr. Wayne Fenton of Yale University School of Medicine, New Haven, CT, U.S.A. Male *spf* mice were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A., and normal male B6C3F1 mice were from Simonsen Laboratories, Gilroy, CA, U.S.A. All animals were fed *ad libitum* on standard chow, and were killed by cervical dislocation.

The following radioactive materials were obtained from ICN, Irvine, CA, U.S.A.: ^3H_2O (sp. radioactivity 100 Ci/ml), [U-^{14}C]sucrose (sp. radioactivity 350 Ci/mol), $NaH^{14}CO_3$ (sp. radioactivity 55 Ci/mol). L-[2,3-^{3}H]Ornithine (sp. radioactivity 20 Ci/mmol) and [^{14}C]carbamoyl phosphate (sp. radioactivity 5.1 Ci/mol) were from New England Nuclear, Boston, MA, U.S.A. Hydrofluor was from National Diagnostics, Somerville, NJ, U.S.A., and Dow Corning silicones were from William F. Nye, Inc., New Bedford, MA, U.S.A. Defatted bovine serum albumin was from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.

Abbreviations used: CPS, carbamoyl-phosphate synthetase (ammonia) (EC 6.3.4.16); OCT, ornithine carbamoyltransferase (EC 2.1.3.3).

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Bovine OCT (sp. activity 650 μmol/min per mg) was purified by the method of Marshall & Cohen [15]. All other reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation and incubation of mitochondria

The livers were removed, rinsed, blotted, weighed and homogenized as described by Raijman & Bartulis [16] in 9 vol. of ice-cold 0.3 M-mannitol/2 mM-Hepes/KOH, pH 7.4. Portions were frozen at −75 °C for enzyme assays. The remainder of the homogenate was diluted 2-fold with the same medium, and mitochondria were prepared as described by Cohen *et al.* [17]. The total volume of mitochondrial suspension obtained was measured, and portions were frozen at −75 °C for enzyme assays. The protein content of the liver homogenates and of the mitochondrial preparations was measured as described by Lowry *et al.* [18], with bovine serum albumin standards.

Mitochondrial incubations were performed in duplicate in a Dubnoff shaking bath at 30 °C. The standard incubation medium contained (final concns.) 50 mM-Tris/HCl, pH 7.4, 15 mM-KCl, 15 mM-$KHCO_3$, 10 mM-NH_4Cl, 5 mM-$MgCl_2$, 5 mM-KH_2PO_4, 2 mM-EDTA, 0.1% defatted bovine serum albumin, 10 mM-succinate, 10 mM-ornithine and 60 mM-mannitol, at a final pH of 7.4. The reactions were started by addition of mitochondria to prewarmed incubation medium, except as noted below. For incubations with uncoupled mitochondria the medium also contained (final concns.) 5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 10 μg of oligomycin/ml, 0.2% ethanol (the vehicle for the oligomycin), 5 mM-*N*-acetylglutamate, 2 mM-ATP, 2.5 units of creatine kinase/ml and 5 mM-phosphocreatine (di-Tris salt); succinate was omitted. For these incubations the uncoupled mitochondria were preincubated for 5 min at 30 °C with the acetylglutamate as described previously [19]. Final incubation volumes were 0.5–1.0 ml, and the final mitochondrial protein concentration was either 2.8–3.8 mg/ml (for incubations designed to measure citrulline and total carbamoyl phosphate synthesis) or 1.3–1.9 mg/ml (for incubations in which ornithine requirements for citrulline synthesis were studied). The reactions were stopped either by the addition of $HClO_4$ (final concn. 1.7 M) for subsequent determination of citrulline in the protein-free supernatant, or as previously described [19] for the measurement of carbamoyl phosphate. Incubations in the absence of ornithine were performed as described by Cohen *et al.* [5], titrations of ornithine for citrulline synthesis in intact mitochondria and the measurement of ornithine in the matrix during citrulline synthesis were done exactly as described by Cohen *et al.* [19], and mitochondrial volumes were measured as previously described [20].

Enzyme assays

The enzymes studied were OCT, CPS, glutamate dehydrogenase, citrate synthase (all matrix enzymes) and β-hydroxybutyrate dehydrogenase (an enzyme of the inner membrane); the activities of these enzymes, and total protein, were measured in liver homogenates prepared from normal and from *spf*^{ash} mice and in mitochondrial suspensions prepared from those homogenates. Estimates of mitochondrial recoveries were based on these enzyme data. In some preparations the activity of cytochrome oxidase, another inner-membrane enzyme, was also determined.

OCT was measured at pH 8.5 as described by Raijman [21]. For determination of the kinetic parameters of the enzyme, ornithine was from 0.05 to 10 mM, and [^{14}C]-carbamoyl phosphate was used at concentrations of 0.01–0.5 mM and specific radioactivities of 2.86–1 μCi/μmol; the invariant substrate was at 5 mM. For the determination of the K_i for phosphate, the latter was at 0, 0.25, 1 or 2 mM, with carbamoyl phosphate as described above. CPS was measured in a medium containing (final concns.) 50 mM-Hepes/KOH, pH 7.4, 1.7 mM-EDTA, 50 mM-NH_4HCO_3, 12 mM-$MgSO_4$, 5 mM-ATP, 5 mM-*N*-acetylglutamate, 5 mM-ornithine, 0.1% defatted bovine serum albumin, 5 mM-phosphocreatine (di-Tris salt), 5 units of creatine kinase/ml and 17 units of bovine OCT/ml, in a total volume of 0.5 ml, at 37 °C. The samples to be assayed were diluted in defatted bovine serum albumin (1 mg/ml) immediately before assay. The reaction tubes, containing everything but ATP, were preincubated at 37 °C for 5 min, and the reactions were started with ATP. They were stopped after either 5 or 10 min by addition of 0.25 ml of 5 M-$HClO_4$. In both the OCT and the CPS assays, citrulline in the deproteinized supernatants was measured by a modification [5] of the method of Ceriotti & Spandrio [22]. The reactions were linear with respect to time and to the amounts of sample assayed; all reactions were done in duplicate.

Glutamate dehydrogenase was assayed by the method of Schmidt [23], citrate synthase as described by Srere [24], by using 5,5′-dithiobis-(2-nitrobenzoic acid), and β-hydroxybutyrate dehydrogenase as described by Bock & Fleischer [25], with a Beckman DU spectrophotometer with a Gilford model 222 power supply, model 410 digital absorbance meter and model 242 recorder. Cytochrome oxidase activity was measured at 30 °C in a medium containing 50 mM-Hepes/KOH, pH 7.4, 15 mM-KCl, 5 mM-$MgCl_2$, 2 mM-EDTA and 120 mM-mannitol. The reactions were started with a mixture of sodium ascorbate (pH adjusted to 6.3–6.5) and *NN*′-tetramethylphenylenediamine, at final concentrations of 20 mM and 4 mM respectively; the final volume was 2 ml. The rate of oxygen decrease was measured with a Yellow Springs Oxygen Monitor, model 53. For all enzyme measurements the samples were thawed and diluted immediately before assay, and several different volumes were used to ascertain that the activity measured was proportional to the amount of sample assayed. The results were analysed for statistical significance by one-way analysis of variance, by using the Epistat programs 'Data-One' and 'Anova'.

RESULTS AND DISCUSSION

Increase in mitochondrial protein per g of liver in *spf*^{ash} mice

There was 12% more protein per g of *spf*^{ash} liver than of normal liver, and the preparation of mitochondria from *spf*^{ash} livers yielded 33% more mitochondrial protein per g of liver than did preparations from normal liver (Table 1). Based on the activity per g of liver of the mitochondrial enzymes measured in both homogenate and mitochondria, the percentage recovery of mitochondrial protein was the same, about 58%, for the mutant and the normal mice. Therefore the calculated

Table 1. Comparison of total liver and mitochondrial protein contents in normal and *spf*^{ash} mice

Results are given as means ± S.E.M. ($n = 4$ for normal and 5 for *spf*^{ash} unless otherwise shown in parentheses).

	Normal	*spf*^{ash}	Change (%)	*P*
Liver protein (mg/g of liver)	183 ± 4.6	205 ± 3.3 (7)	+12	< 0.002
Mitochondrial preparations (mg of protein/g of liver)*	33.8 ± 3.45	45.0 ± 1.27 (8)	+33	< 0.002
Total mitochondrial protein (mg/g of liver)†	57.8 ± 1.27	76.3 ± 3.66	+32	< 0.002
Ratio of specific activities of enzymes in mitochondria and in homogenate	3.18 ± 0.10	2.74 ± 0.13	−14	< 0.025

* From measurements of protein in the mitochondrial suspension and the volume of suspension obtained per g of liver.
† Calculated from line 2 and from the 58% recovery of mitochondria.

total amount of mitochondrial protein per g of *spf*^{ash} liver was also greater (32%) than that of normal liver (Table 1). All of these differences are highly significant. In addition, the *spf*^{ash} mice had relatively large livers; liver weight per unit body weight was about 20% greater than normal (results not shown).

The mean ratio of specific activities of five enzymes in mitochondria to those in the homogenate was different for normal and mutant mice (Table 1) (see below). Mitochondrial protein was 31.6 ± 0.96% (mean ± S.E.M., $n = 4$) and 36.8 ± 1.63% ($n = 5$) of total liver protein in normal and *spf*^{ash} mice respectively, an increase of 17% in the mutant. The increase in the total amount of mitochondrial protein per g of liver and in the size of the liver relative to the body weight may represent adaptive responses to the massive OCT deficiency of these mice.

Alterations in the enzymic profile of *spf*^{ash} mitochondria

The activities of five enzymes in normal and *spf*^{ash} liver homogenates and mitochondria are shown in Table 2. The activity of OCT per g of liver was only 6% of normal, in agreement with the description of this mutant [10,12]. The specific activity of OCT in *spf*^{ash} mitochondria was only 5% of normal. Of the other matrix enzymes studied, CPS and glutamate dehydrogenase were altered in a parallel pattern, whereas citrate synthase behaved somewhat differently. The mitochondrial specific activity of CPS, the enzyme that is functionally most closely related to OCT, was significantly lower, as was that of glutamate dehydrogenase, another enzyme involved in ammonia metabolism. The specific activities were decreased enough that, despite the large increase in total mitochondrial protein, the activities of these two enzymes per g of liver were not very different from normal. For citrate synthase the specific activity in mitochondria was similar to that in normal mice; the overall expansion of the mitochondrial compartment resulted in a significant increase in the total amount of citrate synthase activity in the liver. A third pattern of adaptation was observed for the inner-membrane

Table 2. Mitochondrial enzyme activities in normal and *spf*^{ash} liver homogenates and mitochondrial preparations

For each enzyme, activities are given in terms of units/g of liver (μmol/min per g of liver as measured in homogenates) and specific activities (nmol/min per mg) in homogenates (homog.) and in mitochondria (mit.) prepared from those homogenates. Abbreviations: GDH, glutamate dehydrogenase; CS, citrate synthase; BDH, β-hydroxybutyrate dehydrogenase. The data are given as means ± S.E.M. ($n = 4$ for normal and 5 for *spf*^{ash} unless otherwise shown in parentheses); N.S., not significant.

Enzyme		Normal	Mit.*/Homog.	*spf*^{ash}	Mit.*/Homog.	Change (%)	*P*
OTC	Units/g of liver	309 ± 11.4		17.4 ± 0.55 (7)		−94	< 10^{-6}
	Sp. act. homog.	1696 ± 68		84.4 ± 4.10		−95	< 10^{-6}
	Sp. act. mit.	5286 ± 323	3.12	245 ± 24.4	2.92	−95	< 10^{-6}
CPS	Units/g of liver	8.0 ± 0.45		8.4 ± 0.79 (7)		+5	N.S.
	Sp. act. homog.	43.9 ± 3.92		41.6 ± 5.46		−5	N.S.
	Sp. act. mit.	134 ± 5.9	3.05	111 ± 5.27	2.64	−17	< 0.015
GDH	Units/g of liver	188 ± 3.5		218 ± 9.7 (7)		+16	< 0.05
	Sp. act. homog.	1029 ± 36		1017 ± 65		−1	N.S.
	Sp. act. mit.	3158 ± 39	3.07	2727 ± 129	2.68	−14	< 0.015
CS	Units/g of liver	23.9 ± 1.07		30.6 ± 1.23 (7)		+28	< 0.005
	Sp. act. homog.	130 ± 3.6		146 ± 8.2		+12	N.S.
	Sp. act. mit.	408 ± 6.9	3.14	378 ± 17.4	2.59	−7	N.S.
BDH	Units/g of liver	7.8 ± 0.37		13.0 ± 0.28 (7)		+66	< 10^{-6}
	Sp. act. homog.	42.8 ± 2.47		64 ± 1.78		+50	< 10^{-4}
	Sp. act. mit.	144 ± 7.1	3.36	175 ± 6.6	2.73	+22	< 0.01

* The ratio of specific activities in mitochondria and homogenates.

enzyme β-hydroxybutyrate dehydrogenase. A large increase in its specific activity in the mitochondria, combined with the overall increase in mitochondrial protein per g of liver, resulted in a 66% increase in liver β-hydroxybutyrate dehydrogenase activity. The specific activity of cytochrome oxidase in *spf*^{ash} mitochondria was about 70% of that measured in normal mouse mitochondria (results not shown). These enzymic alterations provide evidence of striking adaptations in the overall biochemical capabilities of the *spf*^{ash} liver mitochondria.

The mitochondrial matrix is a region of very high protein concentration, which has been estimated to be approx. 50% (w/v) [26]. About 25% of matrix protein in liver mitochondria is accounted for by CPS (20%) and OCT (5%) [27,28]. On that basis alone, one would infer that *spf*^{ash} mitochondria, lacking most of the OCT and almost 20% of the CPS, are organelles with a higher ratio of membrane to matrix. The values for β-hydroxybutyrate dehydrogenase demonstrate that the specific activity of at least one inner-membrane enzyme is much higher than normal. The decrease in specific activity of cytochrome oxidase in *spf*^{ash} mitochondria, however, indicates that the alterations are more complex than would result simply from an overall decrease in matrix proteins. Some of the subunits of cytochrome oxidase are mitochondrially coded [29], whereas the other enzymes measured are nuclear-coded proteins [8]. There appear to be major changes from normal in the co-ordination of nuclear and mitochondrial processes in *spf*^{ash} males.

Characteristics of citrulline synthesis in *spf*^{ash} mitochondria

The work described above suggests that the biochemical capabilities of *spf*^{ash} liver mitochondria are greatly altered from those of normal mice. The formation of citrulline for urea synthesis is a major function of mammalian liver mitochondria. Since the *spf*^{ash} OCT deficiency is so marked, it seemed reasonable to expect that those mitochondria should exhibit striking changes in the behaviour of the citrulline-synthesizing system. In the functioning of this system, which has been thoroughly studied in rat liver mitochondria, CPS and OCT operate in a coupled fashion, as follows. When liver mitochondria isolated from rats fed on a normal diet are incubated with a respiratory substrate, ornithine, and all the substrates for CPS, essentially all of the carbamoyl phosphate formed is converted into citrulline. Under special conditions, when the activity of CPS is caused to increase above 40–45 nmol/min per mg, citrulline synthesis becomes limiting and carbamoyl phosphate accumulates [6]. Thus, although the total activity of OCT is normally in about 40-fold excess over CPS [27,28] (Table 2, above), only a small fraction of it is exhibited *in situ*, either because OCT is inhibited or because the transport of ornithine is limiting [19]. When mitochondria are incubated in the absence of ornithine, CPS becomes severely inhibited within a few minutes, displaying an activity less than 10% of that observed in the presence of ornithine [5]. This inhibition does not appear to be caused by product inhibition by carbamoyl phosphate [5]. A functioning OCT appears to be necessary for CPS to display normal activity [6].

These and other observations [5,6,30] suggested that there exists some type of organization or interaction between CPS and OCT. There is also indirect evidence of interaction between CPS and the adenine nucleotide translocator [16], and the channelling of extramitochondrial ornithine to matrix OCT [19,31] indicates that there is direct interaction between this enzyme and an ornithine transporter of the inner membrane. The location of OCT next to the inner membrane has been shown histochemically [32,33], and the association of OCT with the membrane was shown to survive mitochondrial fractionation and to re-occur after purification [33].

Because of the striking deficiency in OCT activity and specific protein in the livers of *spf*^{ash} males, we expected that isolated *spf*^{ash} mitochondria would have a much lower capacity for citrulline synthesis *de novo* than do normal mouse mitochondria, and that they would behave very differently from controls when incubated in the absence of ornithine. We found instead that mitochondria isolated from *spf*^{ash} livers synthesize citrulline at rates not far below those observed with mitochondria from normal mouse liver. In the standard incubation medium, normal mitochondria respiring on succinate formed 33 and 36 nmol/min per mg in two separate experiments, whereas *spf*^{ash} mitochondria formed 25.3 ± 1.47 nmol/min per mg (mean ± S.E.M., $n = 4$). Under these conditions, essentially all of the carbamoyl phosphate formed was converted into citrulline in both normal and mutant mitochondria. Despite the fact that the *spf*^{ash} mitochondria contained only 5% of the normal amount of OCT, the rate of citrulline synthesis at high [ornithine] (10 mM) was compatible with physiological requirements for urea synthesis.

When normal and *spf*^{ash} mouse mitochondria were incubated in the absence of ornithine, the inhibition of CPS was similar in both (Fig. 1), and similar to that previously observed with rat mitochondria [5]. Since the molar ratio of CPS to OCT in *spf*^{ash} mice is about 500 (see [6]), the ornithine effect cannot be a function of some interaction between CPS and OCT.

The allosteric activator of CPS, *N*-acetylglutamate, is not at saturating concentrations in liver mitochondria of animals fed on a normal diet [27,28,34,35]. When isolated rat liver mitochondria are preincubated for 5 min with substrates that support the endogenous synthesis of acetylglutamate (as described by Cohen *et al.* [19]), the activity of CPS during the subsequent incubation can be substantially increased, often by 100%. Unexpectedly, this treatment was not effective with *spf*^{ash} mitochondria, and actually resulted in a 40–50% decrease in CPS activity (results not shown). Addition of 1 mM-pyruvate (to provide an endogenous source of acetyl-CoA) and 2 mM-acetylglutamate (to counter possible leakage of acetylglutamate) to the preincubation mixture did not prevent the loss of activity. These results could reflect alterations in the activity of acetylglutamate synthetase, or in one or more of the required transport systems.

When uncoupled rat liver mitochondria are incubated with acetylglutamate and saturating concentrations of CPS substrates and ornithine, the activity of CPS can be increased to what appears to be $V_{max.}$ [6,30,36]. Under such conditions, the velocity of OCT is insufficient to utilize all of the carbamoyl phosphate formed, and the latter accumulates [6]. When normal mouse mitochondria were incubated in this way, carbamoyl phosphate synthesis was 73 nmol/min per mg, with 64 nmol/min per mg converted into citrulline. For *spf*^{ash} mitochondria the corresponding values were 46 and 44 nmol/min per mg (averages of two separate experiments); the activity of

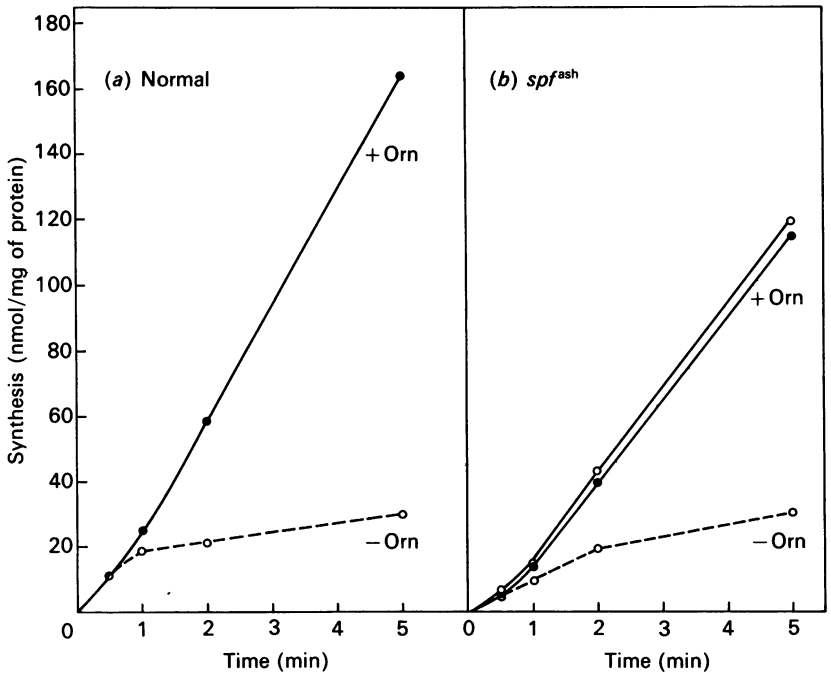

Fig. 1. Carbamoyl phosphate and citrulline synthesis by normal and *spf*^{ash} mitochondria incubated with and without ornithine

The incubations were performed as described in the Materials and methods section and terminated as described by Cohen *et al.* [5]. The data shown are representative of experiments performed with eight separate mitochondrial preparations, four each with normal (*a*) and mutant (*b*) mitochondria. The continuous and the broken lines describe synthesis in the presence and the absence of ornithine respectively. For the *spf*^{ash} incubation the syntheses of both citrulline and total carbamoyl phosphate (including that converted into citrulline) in the presence of ornithine are shown: ●, citrulline; ○, total carbamoyl phosphate.

OCT was sufficient to convert all of the carbamoyl phosphate formed at these rates.

If OCT were normally inhibited in the matrix, our observations would indicate that it is less inhibited in *spf*^{ash} mice than in controls. A likely inhibitor is P_i, which is present at 10–20 nmol/μl in rat liver mitochondrial matrix [37], and is a good competitive inhibitor of OCT [38], with a K_i of 0.25 mM [39]. In two experiments we determined that the K_i for P_i of *spf*^{ash} OCT was about 0.4 mM; therefore, unless the concentration of P_i in *spf*^{ash} mitochondria is several orders of magnitude lower than in rat mitochondria, which is unlikely, the proportionately high rate of citrulline synthesis in *spf*^{ash} mitochondria cannot be explained by a difference in the response of OCT to P_i. We infer from this that inhibition by P_i does not explain the limitation of OCT activity in normal mitochondria. {The K_m value for carbamoyl phosphate was 0.16–0.2 mM for the enzyme from *spf*^{ash} and from normal mice (cf. [12,40]). The K_m for ornithine is dependent on pH; we measured K_m values of 1.16 and 0.16 mM at pH 7.5 and 8.5, respectively, for *spf*^{ash} OCT. These are in good agreement with previously reported values [12,40].}

Effects of [ornithine] on citrulline synthesis by *spf*^{ash} mitochondria

The requirements of normal coupled mouse mitochondria for ornithine were higher than those of rat mitochondria. When CPS functions below about 45 nmol/min per mg, the maximal rate of citrulline synthesis is limited by, and equivalent to, that of carbamoyl phosphate synthesis (see above). Under these conditions external ornithine became saturating for citrulline synthesis at about 0.2 mM in rat mitochondria [19], and at about 0.4 mM in normal mouse mitochondria (Fig. 2*a*). With *spf*^{ash} mitochondria, the shape of the curve describing the response of citrulline synthesis to increasing external ornithine was different (Fig. 2*a*); the rate of citrulline synthesis at a given ornithine concentration was a smaller proportion of the total rate of carbamoyl phosphate synthesis than in normal mitochondria. Similar observations were made when the incubations were done with uncoupled mitochondria preincubated with acetylglutamate to stimulate CPS activity (Fig. 2*b*). Since the K_m for ornithine of OCT from normal and *spf*^{ash} mice is the same *in vitro* ([10,12], above), these data suggest that, at a given external [ornithine], the availability of this substrate to OCT is less in *spf*^{ash} than in normal mitochondria (see below). These observations may have connotations for the actual performance of *spf*^{ash} mitochondria *in vivo*. Ornithine concentration in the liver of normal rats fed on a standard chow diet ranges between 0.15 and 0.45 mM, depending on the time elapsed after feeding (C.-W. Cheung & L. Raijman, unpublished work). Although there are no comparable data for the *spf*^{ash} mouse, it is clear from the results presented here that, despite the adequacy of matrix OCT activity at high [ornithine], the latter may be limiting for citrulline synthesis in *spf*^{ash} liver *in vivo*. This is consistent with the observation by Qureshi *et al.* [41] that *spf*^{ash} males have significant orotic aciduria.

Matrix concentrations of ornithine during citrulline synthesis

In coupled liver mitochondria from rats fed on normal diets, 0.2 mM external ornithine is necessary and sufficient

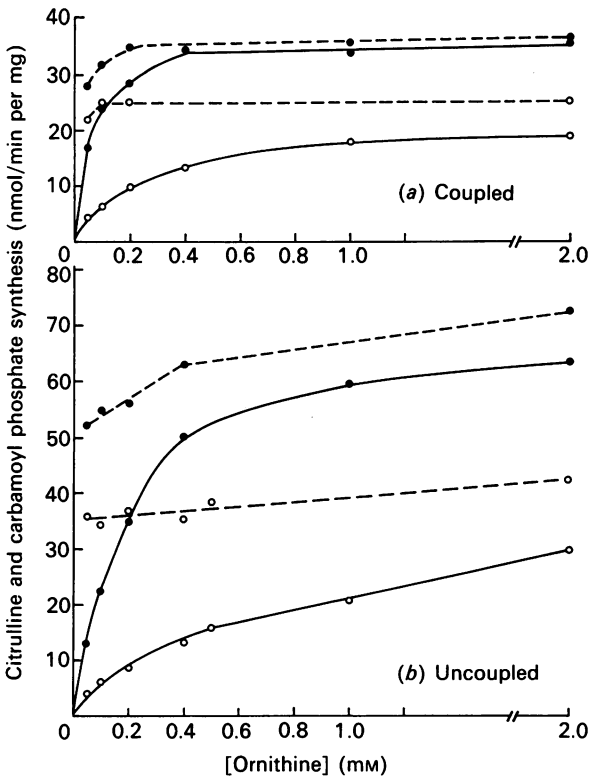

Fig. 2. Citrulline and total carbamoyl phosphate synthesis by normal and *spf*^{ash} mitochondria as a function of external [ornithine]

The incubations were performed exactly as described by Cohen *et al.* (legend to Figs. 1A and 1C in ref. [19]). The values shown are the averages of two closely agreeing experiments. (*a*) Coupled mitochondria respiring on succinate; (*b*) uncoupled mitochondria preincubated with acetylglutamate. The continuous lines describe citrulline synthesis, and the broken lines total carbamoyl phosphate synthesis (including that converted into citrulline). ●, Normal mouse mitochondria; ○, *spf*^{ash} mitochondria.

to support conversion of carbamoyl phosphate into citrulline at the highest rates observed in these preparations (i.e. about 45 nmol/min per mg). Yet ornithine is not detectable in the matrix even when external [ornithine] is as high as 0.5–1 mM [19]. It was shown in those studies that external ornithine is channelled to matrix OCT [19,31]. In normal mouse mitochondria there was no measurable ornithine in the matrix at external [ornithine] of 0.1 mM, whereas at 0.4 mM external ornithine there was 0.24 nmol/μl of matrix volume. (Matrix concentrations of citrulline were 1.18 and 1.58 nmol/μl, and rates of citrulline synthesis were 27 and 35 nmol/min per mg, at 0.1 mM- and 0.4 mM-ornithine respectively.) In contrast, in *spf*^{ash} mitochondria incubated under identical conditions, ornithine was found in the matrix: 0.05, 0.16, 0.18 and 0.25 nmol/μl of matrix volume were measured at external [ornithine] of 0.05, 0.1, 0.2, and 0.4 mM respectively. (Matrix citrulline was 0.56, 0.71, 1.10 and 1.40 nmol/μl, and total citrulline synthesis was 5, 8, 13 and 17 nmol/min per mg at the same [ornithine] respectively.)

The affinity of OCT from normal and *spf*^{ash} mice for ornithine is the same, yet at a given external [ornithine] the *spf*^{ash} OCT *in situ* is much less active than the normal (see above and Fig. 2). Since ornithine was found in the matrix of the mutant mitochondria, it is unlikely that transport was limiting for OCT. Taken together, these observations suggest that *spf*^{ash} mitochondria do not use external ornithine as efficiently as do normal mitochondria; it is possible that the interaction between OCT and the inner membrane observed in normal mitochondria is disrupted in the mutant.

Mitochondrial volumes

Mitochondrial volumes measured in normal and *spf*^{ash} mitochondria were comparable; matrix volumes of 1.29–1.50 μl/mg of protein were obtained in both systems when 0.77–0.64 mg of protein was centrifuged. The effect of the amount of protein centrifuged on measured and calculated mitochondrial spaces (total water, sucrose-permeable and matrix) was the same as reported for the rat (results not shown) (see Fig. 4 of ref. [20]).

Limited studies with isolated liver mitochondria from *spf* mice

The livers of *spf* mice have increased amounts of a variant OCT protein with a decreased affinity for ornithine [9,10]. Isolated *spf* liver mitochondria incubated in the standard medium (with 10 mM-ornithine) synthesized carbamoyl phosphate and citrulline at a rate of 33 nmol/min per mg; essentially all of the carbamoyl phosphate formed was converted into citrulline. Thus in this mutant the residual OCT is capable of catalysing citrulline synthesis at normal rates if ornithine is present at high concentrations. Carbamoyl phosphate synthesis became inhibited in the absence of ornithine, exactly as observed with normal mouse mitochondria. The specific activity of CPS in *spf* liver was 43% higher than in normal mouse liver, in agreement with previous reports [41,42]. This is different from what occurs in *spf*^{ash} mice, and may be related to the increased amounts of mutant OCT protein in *spf* liver.

Conclusions

The enzyme data described above show that what appears to be a single mutation in *spf*^{ash} mice results in an expanded mitochondrial population greatly altered in its biochemical characteristics, and presumably in its attendant capabilities. In fact, the experiments using mitochondria provide a picture of a population only subtly altered from normal in its abilities to carry out citrulline synthesis *de novo*, the function one would expect to be most affected. With all substrates saturating, the rate of carbamoyl phosphate synthesis in *spf*^{ash} mitochondria was 30% lower than normal, reflecting the decreased specific activity of CPS, but the CPS–OCT system behaved no differently from normal. That is, at high external [ornithine] the 5% residual OCT activity was sufficient to handle all of the carbamoyl phosphate formed (at a very respectable rate). The deficiency of the system was observed only when ornithine was at concentrations below 1–2 mM.

These studies on *spf*^{ash} mice clarify some aspects of the functioning of the CPS–OCT system in normal rat and mouse mitochondria. In these, the maximal velocity of citrulline synthesis ever observed is a very small fraction (about 1–2%, see Table 2) of the $V_{max.}$ of OCT measured in lysed mitochondria. As previously pointed out [19], this could be the result either of severe inhibition of OCT

in situ or of limitation of citrulline synthesis by ornithine transport. It was suggested [6] that OCT *in situ* might be inhibited by P_i, but the studies described here seem to eliminate this possibility; if OCT in normal mitochondria is inhibited by other mechanisms, these must not affect the enzyme in *spf*^{ash} mitochondria. It is simpler to postulate that ornithine transport limits citrulline synthesis at maximal rates of carbamoyl phosphate synthesis. However, this must be reconciled with two facts: first, that under exactly those conditions, ornithine is present in the matrix at high concentrations (about 20 nmol/μl of water) [6]; second, that at the high external concentrations of ornithine at issue, 5–10 mM, ornithine bypasses this transporter [3]. It must be postulated, therefore, that at high external [ornithine] two or more poorly mixing pools of ornithine exist in the matrix. Evidence consistent with this was given in ref. [19].

Finally, it is now clear that the stimulation of carbamoyl phosphate synthesis by ornithine *in situ* does not require protein–protein interaction between CPS and OCT [5,6].

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