INCREASE OF ORNITHINE TRANSCARBAMYLASE PROTEIN IN SPARSE-FUR MICE WITH ORNITHINE TRANSCARBAMYLASE DEFICIENCY

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1. Introduction

Ornithine transcarbamylase (OTCase, EC 2.1.3.3) a mitochondrial enzyme of the urea cycle catalyses the formation of citrulline from ornithine and carbamoyl phosphate. It has been purified from liver in 3 mammalian species: bovine [1], human [2] and rat [3,4] but not from mouse. We decided to purify mouse OTCase because the mouse is the only mammalian species in which two strains with an OTCase mutation have been described: sparse-fur (spf) and sparse-fur with abnormal skin and hair (spf ash) 5,6]. These two X-linked mutations are easily maintained during breeding through several generations and are good models for the study of OTCase mutations as compared to those observed in humans.

We have been especially interested in mouse spf in which the OTCase activity exhibits peculiar properties: at pH 8 which is the optimum pH of the normal enzyme, the spf OTCase activity is decreased by 75% while at pH 9 it is increased by 150% as compared to control OTCase measured at its optimum pH (pH 8).

The aim of our work was to answer the question raised by this observation: is this increase of OTCase activity at pH 9 only due to a physico–chemical modification of the enzyme or is it secondary to an increased amount of this protein? This latter possibility would imply that when a mutation affects the quanlitative function of an enzyme of the urea cycle, a mechanism would be triggered resulting in an increased amount of the defective protein.

We describe here the purification of OTCase from mouse liver and a quantitative assay of this enzyme by an immunological procedure [7] using a specific antiserum raised in rabbit. The properties of normal and spf mouse livers are compared.

2. Materials and methods

2.1. *Animal breeding*

All the mice mentioned are either (+/Y) or (spf/+) males kindly supplied by I. A. Qureshi and J. Letarte [5]. A standard diet with 20% protein was used. Sparse-fur and normal males were both found to survive after weaning on this diet but spf males were always hypotrophic as compared to normal males.

2.2. *Purification of normal mouse liver OTCase*

Affinity chromatography with the immobilized transition state analog δ-*N*-(phosphonacetyl)-L ornithine (δ-PALO) was done [4]. δ-PALO was synthesized as in [8] and coupled to epoxy-activated Sepharose 6B on a shaker at 37°C overnight in 0.1 M NaOH [9].

Ten mouse livers (13.8 g) were homogenized in 100 ml buffer containing 0.5% (w/v) Triton X-100, 10 mM NaHepes (pH 7.4) and 2 mM dithiothreitol. After stirring for 30 min at 4°C the homogenate was centrifuged at 10 000 × *g* for 10 min and the supernatant was passed through a column of δ-PALO Sepharose 6B (1 × 10 cm). The column was washed [4] and the OTCase was eluted with 10 mM NaHepes (pH 7.4) and 10 mM carbamoyl phosphate. The fractions containing the OTCase were pooled (15 ml), and the purity of the preparation was checked by electrophoresis on sodium dodecyl sulfate (SDS)–polyacrylamide gel [10].

2.3. *Determination of molecular mass* (M_r)

The M_r was determined by chromatography on Sephadex G-200 with: uricase (122 000), serum albumin (67 000), ovalbumin (43 000) and trypsin (23 000) as markers.

Table 1
Purification of ornithine transcarbamylase from mouse liver

Fraction	Total activity (units)	Total protein (mg)	Spec. act. (units/mg)	Yield (%)	Purification
Triton X-100 supernatant from 10 livers	2640	2016	1.3	100	
δ-PALO Sepharose 6B fraction	2020	6.2	326	76.5	250

The subunit M_r was determined by SDS–polyacrylamide gel electrophoresis as in [11] using: phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soya bean trypsin inhibitor (20 100) and lactalbumin (14 100) as markers.

2.4. *Immunological procedure*

Antibodies against mouse OTCase were raised by injecting rabbits once a week for three consecutive weeks with 0.8 mg purified enzyme mixed with Freund's adjuvant. Ten days later, the blood was collected, centrifuged and the serum analyzed by the Ouchterlony method [12] for its content in OTCase antibodies. Immunoelectrophoresis [13] was used to demonstrate the monospecificity of the antiserum.

To measure the amount of OTCase protein, Mancini radial immunodiffusion was performed [7]. A straight-line relationship exists between the antigen concentration and the area of the terminal immunoprecipitate.

2.5. *Enzymatic analysis*

OTCase activity was assayed according to [14] with 0.2 M triethanolamine–HCl buffer (pH 7–10) and the two substrates ornithine (5 mM) and carbamoyl phosphate (5 mM). The activity is defined as the amount of citrulline formed in μmol . min^{-1} . mg protein^{-1}. Citrulline was assayed by an automated colorimetric method [15].

2.6. *Preparation of homogenates and mitochondria*

Liver extracts from normal and spf mice were homogenized in 14 vol. cold 0.1% cetyltrimethylammonium bromide and used directly for the determination of protein concentration and Mancini radial immunodiffusion. Liver mitochondria were isolated from normal and spf mice as in [16] in 0.27 M saccharose and 2 mM Tris–HCl (pH 7.4).

3. Results and discussion

3.1. *Purification and molecular weight determination of mouse ornithine transcarbamylase*

The purification procedure of normal mouse OTCase is given in table 1. In 6 different experiments the yield was 75–85%, and spec. act. 280–330 μmol . min^{-1} . mg OTCase^{-1}. The purity of the OTCase preparations was checked by acrylamide gel electrophoresis (fig.1A). The M_r was 98 000 as determined by Sephadex G-200 filtration (not shown) and the subunit M_r was 36 000 (fig.1B). These results agree with a trimeric structure reported for rat [3,4], bovine [1] and human [2] OTCase.

Using the purified mouse OTCase we obtained a rabbit monospecific antiserum (fig.2). After electrophoresis of mouse liver homogenate and of purified OTCase, only one precipitation arc was observed by development with a rabbit antiserum raised against purified mouse OTCase; there is no precipitation arc

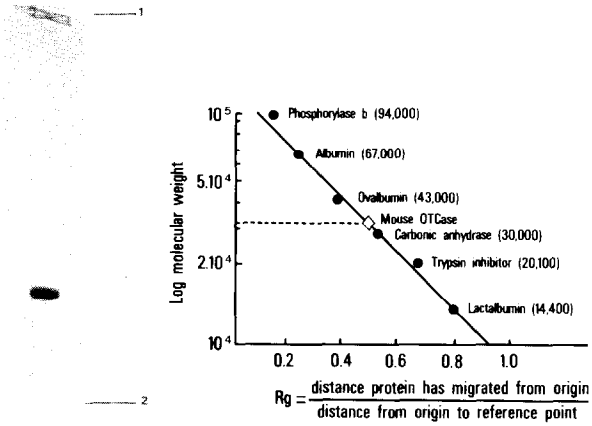

Fig.1. SDS–polyacrylamide gel pattern of purified OTCase of normal mouse: (A) 13 μg protein were applied onto the gel at the level of line 1. The dye front is indicated by line 2; (B) purified OTCase (8 μg) and markers were subjected to electrophoresis [10].

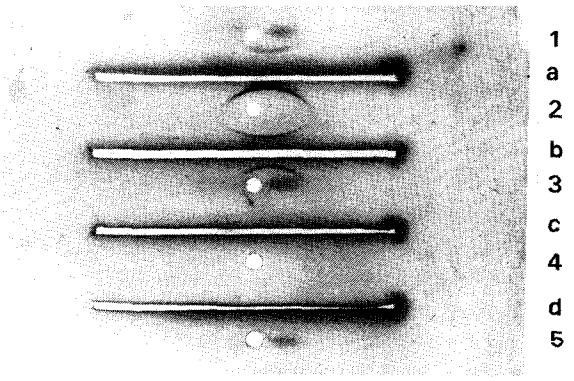

Fig.2. Immunoelectrophoresis [11] of mouse liver homogenate (1 mg protein in wells 1,3,5) and of purified mouse OTCase (2 μg in wells 2,4). After electrophoresis (10 V/cm, 2 h) the precipitation pattern was developed with a rabbit antiserum raised against purified mouse OTCase (a,b) and with control rabbit antiserum (c,d).

after development with control rabbit serum.

The purification of spf mouse OTCase by the same procedure gives lower yields (~10%); very minor impurities were detectable when the preparation was checked by acrylamide gel electrophoresis (not shown).

3.2. *Determination of the amount of OTC protein in liver homogenates and mitochondria from normal and spf mice*

Radial immunodiffusion clearly demonstrates (fig.3) that there is more OTCase cross-reactive material (CRM) in spf than in wild-type hepatic homogenate. In 5 different experiments, we always found spf CRM/control CRM ratios of 1.1–1.5 ($\bar{m}$ = 1.2 ± 0.06 SEM). The problem was to define which form of the molecule results in the increase of CRM observed. OTCase is synthesized as a larger cytosolic precursor which may be imported into mitochondria concomitantly with post-translational processing to yield mature OTCase [17,18]. When radial immunodiffusion was performed with isolated mitochondria, the same ratio for spf CRM/control CRM was found [1,2]. This indicates that mature intra-mitochondrial OTCase is increased in spf mice and rules out the possibility of a mitochondrial membrane defect in the transport of spf pre-OTCase from the cytosol.

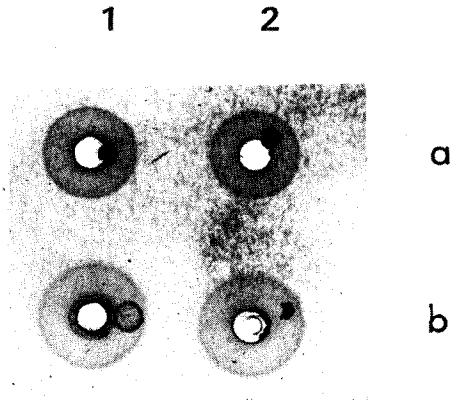

Fig.3. Radial immunodiffusion of liver homogenate and mitochondria from spf and normal mouse; identical amounts of protein (1 mg liver extracts (1) and 0.2 mg mitochondria (2)) from normal (a) and spf (b) mouse were placed on an agarose gel containing anti-mouse OTCase (70 μg/ml).

3.3. *Determination of OTCase activity as a function of pH*

Kinetic abnormalities of spf OTCase activity as a function of pH in liver homogenates have been reported [5,6]. Optimum activity is found at pH 9. At this pH, the spf OTCase activity is higher than the activity of the normal enzyme at pH 8, its optimum pH. We found that the ratio spf OTCase at pH 9/control OTCase at pH 8 was 1.45 ± 0.05 SEM. The kinetic abnormalities of partially purified spf OTCase are the same as in total homogenate (fig.4). Because spf OTCase was not completely purified, the true value of the enzyme concentration was determined by Mancini immunodiffusion. The specific activity at pH 9 was 400 ± 30 μmol . min^{-1} . mg spf $OTCase^{-1}$, while the optimum (pH 8) specific activity of purified normal enzyme was 300 ± 30 μmol . min^{-1} . mg $OTCase^{-1}$.

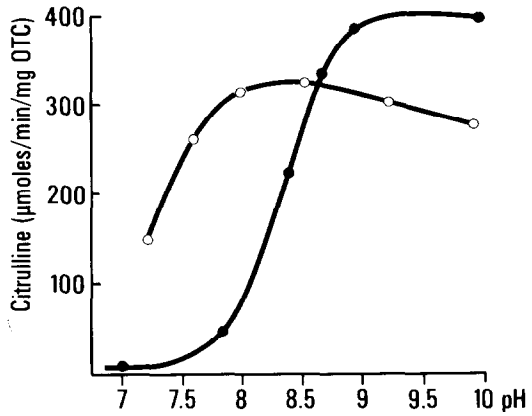

Fig.4. Variations of the activities of normal and spf mouse OTCase as a function of pH. The activity is expressed in μmol citrulline formed . h^{-1} . mg $OTCase^{-1}$ as measured by radial immunodiffusion: (○) normal OTCase; (●) spf OTCase.

4. Conclusion

The high activity of spf OTCase at pH 9 observed in crude homogenates appears to be the result of two kinds of events:

(1) An enhancement in the amount of OTCase protein in liver mitochondria as demonstrated by immunological measurements. It must therefore be assumed that a mechanism enhances the amount of mutant OTCase which tends to counter-balance the decrease in its catalytic activity at the physiological pH of the cell.

(2) The specific activity is also enhanced at high pH resulting in modifications of the physico-chemical properties of the mutant enzyme which remain to be determined.

The analogy between spf OTCase and human pathology must be underlined. We have recently described [19] a patient with greater activity than the normal at pH 9 and a parallel enhancement of CRM as compared to normal human liver. Further investigations on mice should give a better knowledge of the molecular basis of this human mutation.

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