Biochem. J. (1990) 272, 671–675 (Printed in Great Britain)

Effect of level of dietary protein on arginine-stimulated citrulline synthesis

Correlation with mitochondrial *N*-acetylglutamate concentrations

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Increases in dietary protein have been reported to increase the rate of citrulline synthesis and the level of *N*-acetylglutamate in liver. We have confirmed this effect of diet on citrulline synthesis in rat liver mitochondria and show parallel increases in *N*-acetylglutamate concentration. The magnitude of the effect of arginine in the suspending medium on citrulline synthesis was also dependent on dietary protein content. Mitochondria from rats fed on a protein-free diet initially contained low levels of *N*-acetylglutamate, and addition of arginine increased the rate of its synthesis. Citrulline synthesis and acetylglutamate content in these mitochondria increased more than 5-fold when 1 mM-arginine was added. A diet high in protein results in mitochondria with increased *N*-acetylglutamate and a high rate of citrulline synthesis; 1 mM-arginine increased citrulline synthesis in such mitochondria by only 36%. The concentration of arginine in portal blood was 47 μM in rats fed on a diet lacking protein, and 182 μM in rats fed on a diet containing 60% protein, suggesting that arginine may be a regulatory signal to the liver concerning the dietary protein intake. The rates of citrulline synthesis were proportional to the mitochondrial content of acetylglutamate in mitochondria obtained from rats fed on diets containing 0, 24, or 60% protein, whether incubated in the absence or presence of arginine. Although the effector concentrations are higher than the K_a for the enzymes, these results support the view that concentrations of both arginine and acetylglutamate are important in the regulation of synthesis of citrulline and urea. Additionally, the effects of dietary protein level (and of arginine) are exerted in large part by way of modulation of the concentration of acetylglutamate.

INTRODUCTION

Protein is the major source of HCO_3^- and NH_4^+ in typical rat (and human) diets, and urea synthesis functions to dispose of these products of protein catabolism. The level of protein in the diet has been shown to affect the rate of urea synthesis in perfused liver (Saheki *et al.*, 1977) and the rate of citrulline synthesis in isolated mitochondria (McGivan *et al.*, 1976). The mechanism by which dietary protein affects the rate of urea synthesis has not been established and remains a subject of debate.

Carbamoyl phosphate synthetase I (EC 6.3.4.16) is found in liver mitochondria of all ureotelic animals, and may be considered to be the first enzyme of urea synthesis. *N*-Acetylglutamate is an obligatory modifier of this enzyme. The rate of synthesis of acetylglutamate is, in turn, modulated by the concentration of arginine, a positive modifier of acetylglutamate synthase. These effects suggest a regulatory cascade similar to amplifying cascades observed in other areas of metabolic regulation (Stadtman & Chock, 1978). The physiological significance of this potential regulatory cascade has been the subject of much discussion (Stewart & Walser, 1980; Lund & Wiggins, 1984; Meijer & Verhoeven, 1984; Kawamoto *et al.*, 1985).

Changes in arginine concentrations associated with dietary protein intake may increase *N*-acetylglutamate synthesis, as proposed by Shigesada *et al.* (1978). Stewart & Walser (1980) argue against that possibility, and propose that an increase in the concentration of glutamate, rather than arginine, is responsible for the increased rate of urea synthesis that accompanies high protein intake. To investigate the relevance of arginine as a regulatory signal in controlling citrulline synthesis and to understand the relation of dietary protein intake and the rate of citrulline synthesis, we have studied the effect of arginine on liver mitochondria isolated from rats fed on various protein-containing diets.

EXPERIMENTAL

Enzymes and reagents

Urease type VI, ATP, pyruvate, fatty-acid-free BSA, ornithine transcarbamoylase and all amino acids were from Sigma Chemical Co. Rat liver carbamoyl phosphate synthetase was purified as described as described by Powers (1981). The various protein test diets were obtained from Ralston Purina (St. Louis, MO, U.S.A.). These test diets were a purified synthetic diet providing (by wt.) 10% fat, 3% fibre, 2% RP vitamin mixture and 5% RP mineral mixture. The protein content was as indicated, and energy balance was maintained by varying the carbohydrate fraction. Fluoraldehyde reagent for amino acid derivative formation and the Bradford dye reagent were from Pierce Chemical Co. The CO_2/O_2 gas mixtures were purchased from Matheson Gas Products. All other chemicals were of reagent grade or better.

Dietary treatments

Male Sprague–Dawley rats (180–230 g) were maintained on standard rat chow. Starting at 08:00 on the day before they were to be used, rats were fed for 24 h *ad lib.* on a protein-free, 5%-protein, 24%-protein or 60%-protein diet. The amount of food consumed was 10–15 g per rat, and was similar for each test diet group. Food was withheld for 2 h before rats were killed.

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Mitochondrial incubations

Hepatic mitochondria were isolated in 0.3 M-mannitol/2 mM-Hepes/1 mM-EGTA/0.1% fatty-acid-free BSA, pH 7.4, by the method of Myers & Slater (1957) and adjusted to a final concentration of 30 mg of mitochondrial protein/ml. The stimulation of citrulline synthesis after incubation with arginine has been demonstrated by Cheung & Raijman (1981), using a two-stage incubation, in which the amount of *N*-acetylglutamate produced in the first stage is assayed by its stimulation of citrulline synthesis in the second stage. In our modification of this method, mitochondria were incubated (Stage I) in a final volume of 1 ml containing 73 mM-KCl, 24 mM-$KHCO_3$, 45 mM-mannitol, 7.5 mM-KH_2PO_4, 7.5 mM-$MgCl_2$, 3 mM-EDTA, 3 mM-ATP, 10 mM-pyruvate, 10 mM-glutamate, 12 units of urease/ml and arginine as indicated. The incubation medium was equilibrated with O_2/CO_2 (19:1) at 37 °C to give a final pH of 7.4. To support citrulline synthesis during Stage II, ornithine, NH_4Cl and succinate (as an energy source) were added in 0.5 ml in amounts calculated to give a final concentration of 10 mM of each in the incubation mixture. Incubations were carried out for an additional 10 min and terminated by transferring 0.8 ml of the assay mixture to 0.2 ml of 30% (w/v) trichloroacetic acid. Protein was removed by centrifugation. Results are expressed as means ± S.E.M. ($n = 3$).

Blood sampling

Rats were anaesthetized with sodium pentobarbital (80 mg/kg). The portal vein was cannulated with a 21-gauge needle, and blood was collected into a heparinized tube containing 0.3 M-$ZnSO_4$. The sample was then neutralized with $Ba(OH)_2$, and protein was removed by centrifugation.

Assays for metabolites

A colorimetric assay was used for the determination of citrulline (Geyer & Dabich, 1971). Because this method detects urea as well as citrulline, appropriate controls using urease were run.

N-Acetylglutamate was determined by the kinetic assay of Cohen (1984). Mitochondrial *N*-acetylglutamate content was determined by incubating a protein-free extract of the mitochondrial incubations with purified carbamoyl phosphate synthetase. Carbamoyl phosphate production was determined by the incorporation of $H^{14}CO_3^-$ into citrulline, by using a coupled enzyme assay with an excess of ornithine and ornithine transcarbamoylase. The rate of citrulline synthesis in this purified assay is directly proportional to the amount of *N*-acetylglutamate and is linear up to 20 mM. It was necessary to purify rat liver carbamoyl phosphate synthetase to decrease the *N*-acetylglutamate background in the assay. Potassium phosphate buffer was used, since Lund & Wiggins (1987) have shown carbamoyl phosphate synthetase to be inhibited by Tris and Hepes.

Protein was assayed by the method of Bradford (1976), with BSA as the standard.

Amino acids were estimated by reversed-phase h.p.l.c. essentially as described by Jones & Gilligan (1983). *o*-Phthaldialdehyde-derivatives of amino acids were separated on a column of Nova-Pak C-18 (4 μm particle size) (15 cm × 3.9 mm) (Waters–Millipore). The amino acid derivatives were detected fluorimetrically.

RESULTS

To ascertain the effect of arginine on *N*-acetylglutamate synthesis and subsequent citrulline synthesis, we have used a slightly modified version of the two-stage incubation technique of Cheung & Raijman (1981). In the first stage, mitochondria were incubated with glutamate and pyruvate, the substrates for *N*-acetylglutamate synthesis and, where indicated, with arginine, the allosteric modifier of acetylglutamate synthase. In Stage II the necessary substrates to support citrulline synthesis (ornithine, NH_4^+ and succinate) were added. Mitochondrial carbamoyl phosphate production has an absolute requirement for the cofactor *N*-acetylglutamate; thus citrulline synthesis in the two-stage incubation is related to the content of acetylglutamate at the end of Stage I. By this technique it is possible to study in intact mitochondria the effect of arginine on citrulline production, mediated through *N*-acetylglutamate.

Citrulline synthesis in the absence of added arginine was greater in mitochondria from rats fed on a diet with higher contents of protein (Fig. 1, [arginine] = 0). Mitochondria isolated from rats fed on a protein-free diet synthesized citrulline at a rate of 11.2 ± 0.4 nmol/min per mg. Corresponding rates in mitochondria from rats fed on diets with normal (24%) and high (60%) contents of protein were 40.8 ± 0.9 and 57.3 ± 0.4 respectively.

Dietary protein content affects not only the rate of citrulline production but also the response of citrulline production to exogenously supplied arginine (Fig. 1). The relatively high rate of synthesis in mitochondria from rats fed on a high-protein diet increased by only 36% when 1 mM-arginine was added to the suspending medium. However, mitochondria from rats fed on a protein-free diet were highly sensitive to changes in arginine concentrations. The rate of citrulline synthesis increased by 270% when 0.2 mM-arginine was added, and by 430% on addition of 1 mM-arginine. Corresponding increases in mitochondria from rats fed on a diet containing 24% protein were 41% and 69%.

These observations are consistent with the hypothesis that the difference in rates of citrulline synthesis resulted primarily from differences in *N*-acetylglutamate concentration. That relationship was tested in similar experiments by removing a sample at the end of Stage-I incubation for assay of *N*-acetylglutamate content. The remainder of the incubation mixture was used as before to support citrulline synthesis (Stage II). In this experiment, mitochondria from rats on all three diets were used, and were incubated at arginine concentrations ranging from 0 to 1 mM. The results (Fig. 2) show that the rate of citrulline synthesis during Stage-II incubation was directly proportional to the amount of acetylglutamate in the mitochondria at the beginning

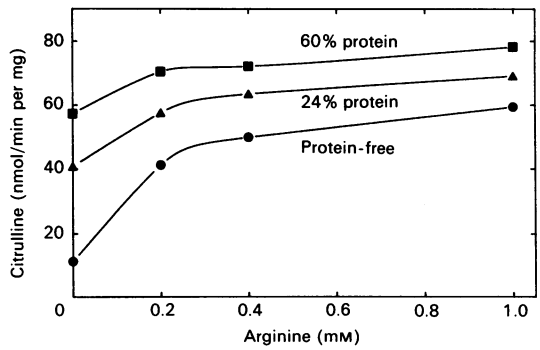

Fig. 1. Citrulline synthesis in rat liver mitochondria as a function of dietary protein content: effect of arginine concentration in the suspending medium

Mitochondria isolated from rats fed on diets containing 0% (●), 24% (▲) or 60% (■) protein were incubated with various concentrations of arginine by the two-stage incubation method described in the Experimental section. The length of the Stage-I incubation was 5 min.

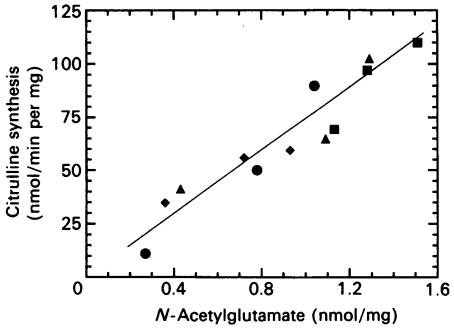

Fig. 2. Correlation between mitochondrial *N*-acetylglutamate content and citrulline synthesis

In separation experiments, mitochondria from rats fed on a protein-free diet (●), a 5%-protein diet (◆), a 24%-protein diet (▲), or a 60%-protein diet (■) were incubated with various concentrations of arginine (0–1 mM). After 5 min, the amount of *N*-acetylglutamate present was determined and the rate of citrulline synthesis monitored for an additional 10 min after addition of NH_4Cl, ornithine and succinate to give final concentrations of 10 mM each.

of that incubation. The linearity of the relationship plotted in Fig. 2 indicates that the level of acetylglutamate was the factor primarily limiting the rate of citrulline synthesis in these mitochondria, whatever the diet of the animals from which they were obtained or the arginine concentration in the suspending medium. That observation strongly supports the conclusion that the level of dietary protein and the concentration of arginine affect the rate of urea synthesis primarily by way of their effects on the concentration of acetylglutamate, rather than directly.

The linear correlation between the rate of citrulline synthesis and *N*-acetylglutamate content (Fig. 2) allows the rate of citrulline synthesis in the two-stage incubation to be used in the estimation of mitochondrial *N*-acetylglutamate content. The rate of citrulline synthesis in the absence of Stage-I incubation (Fig. 3, zero time) reflects the amount of *N*-acetylglutamate initially present in the mitochondria. The initial levels of *N*-acetylglutamate in mitochondria from rats on the three diets, extrapolated from the correlation of citrulline synthesis and *N*-acetylglutamate concentrations (Fig. 2), expressed as nmol/mg of protein, were: protein-free, 0.2; 24% protein, 0.8; 60% protein, 1.0.

It should be noted that the vertical scale of Fig. 2 reflects the concentration of acetylglutamate that is in the mitochondria and is thus available to affect the rate of synthesis of carbamoyl phosphate, whereas the horizontal scale reflects total acetylglutamate, including any that had been lost to the suspending medium. A very low rate of leakage during isolation and storage of the mitochondria is indicated by these observations, confirming reports by McGivan *et al.* (1976) and others that diet-induced differences in mitochondrial acetylglutamate concentration persist during isolation. An alternative but unlikely possibility is that the same fraction had been lost from mitochondria in each assay, in which case our estimations of the levels of mitochondrial acetylglutamate would be high by a constant factor, although comparisons would be valid. The net rate of *N*-acetylglutamate formation in mitochondria (synthesis minus degradation) during the first incubation is indicated by the initial slope of the curves in Fig. 3. Mitochondria from rats on a protein-free diet had a low initial level of *N*-acetylglutamate, which was maintained during incubation in the absence of arginine. As the amount of dietary protein increased, the initial level of *N*-acetylglutamate also increased, and addition of arginine to the incubation medium was necessary to maintain the initial content of acetylglutamate. Apparently, in the absence of added arginine, mitochondria with

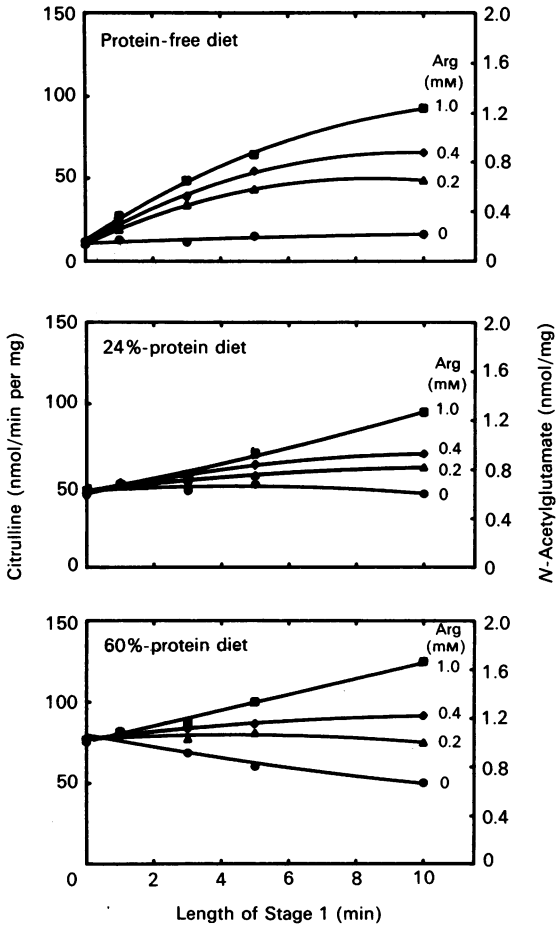

Fig. 3. Rate of *N*-acetylglutamate formation as a function of arginine concentration and dietary protein content

Mitochondria isolated from rats fed on diets containing 0%, 24% or 60% protein were incubated for various times with 10 mM-glutamate, 10 mM-pyruvate and arginine as indicated. At the end of the Stage-I incubation, substrates for citrulline synthesis were added to final concentrations of 10 mM-ornithine, 10 mM-NH_4Cl and 10 mM-succinate, and this Stage-II incubation was carried out for an additional 10 min. *N*-acetylglutamate content is estimated from Fig. 2.

elevated levels of acetylglutamate were unable to synthesize it at a rate sufficient to balance its leakage out of the mitochondria under the conditions of the incubation (Fig. 3, 60%-protein diet). The retention of acetylglutamate inside mitochondria during purification contrasts with the significant rate of decline in its concentration during this incubation, and with estimates for the half-life of mitochondrial acetylglutamate *in vivo* in the range of 15–30 min (Shigesada *et al.*, 1978; Meijer & van Woerkom, 1982). A possible explanation of this apparent paradox is the report (Meijer *et al.*, 1982) that mitochondria lose acetylglutamate to the medium only in the presence of an oxidizable substrate. However, under somewhat different conditions, Cohen & Cheung (1984) observed that addition of dinitrophenol to uncouple ATP synthesis did not significantly affect the exit of acetylglutamate from mitochondria.

Dietary protein content also has a pronounced effect on the portal concentration of arginine (Fig. 4). When rats were fed on a protein-free diet for 24 h before being killed, the concentration of arginine in the portal vein was 47.3 ± 2.8 μM. When the diet contained 60% protein, the portal arginine concentration (182.1 ± 6.1 μM) was nearly 4 times as large.

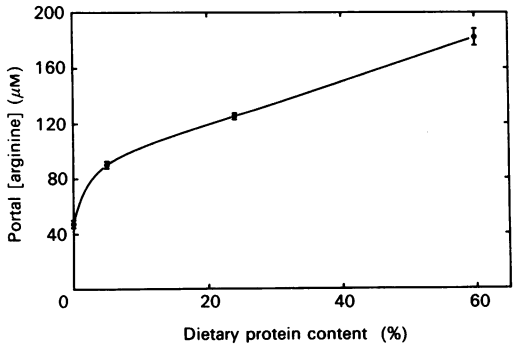

Fig. 4. Portal arginine concentration as a function of dietary protein intake

Rats were fed on various protein diets, and the concentration of arginine in the portal blood was determined by h.p.l.c. Each point represents the mean ± S.E.M. for $n = 3$.

DISCUSSION

Cheung & Raijman (1981) reported a 29% increase in citrulline synthesis by mitochondria from rats fed on a normal protein diet when 1 mM-arginine was added to the incubation medium. We confirm their result (Fig. 1) and show that the sensitivity of mitochondrial citrulline synthesis was dependent on prior dietary treatment (Figs. 1 and 3). Arginine affected the rate of citrulline synthesis appreciably in mitochondria from rats fed on a protein-free diet, but only weakly in mitochondria from animals on a high-protein diet (Fig. 1). This difference appears to result from differences in the endogenous levels of the effector *N*-acetylglutamate.

The effect of dietary protein and arginine on mitochondrial citrulline synthesis seems to be mediated through alterations in *N*-acetylglutamate content (Figs. 2 and 3). The rate of citrulline synthesis is directly proportional to *N*-acetylglutamate concentration (Fig. 2) whatever the level of dietary protein or concentration of arginine in the incubation medium. This result suggests that arginine is a major indicator of dietary protein and that its effects on urea synthesis are exerted primarily by way of acetylglutamate.

The level of acetylglutamate that we measured in mitochondria (in the range 0.5–1.5 nmol/mg of protein) is similar to those reported by others (Meijer & van Woelkom, 1978; Aoyagi *et al.*, 1979; Rabier *et al.*, 1982; Morita *et al.*, 1982; Cohen & Cheung, 1984), and also is not inconsistent with the estimate by Lund & Wiggins (1984) of 1 mM or more in mitochondria, based on whole-cell measurements. As the last workers point out, such concentrations are well above the K_a value of about 0.1 mM reported by several workers (Elliott & Tipton, 1974; Pierson & Brien, 1980; Kamemoto, 1982; Lof *et al.*, 1983), which would seem to argue against an active role for acetylglutamate in regulation of carbamoyl phosphate synthesis. Variations in assay conditions have since led to even lower estimates of K_a: 17 μM when Mn^{2+} replaced Mg^{2+} (Cohen, 1984) and 11 μM when phosphate buffer was used instead of Hepes or Tris (Lund & Wiggins, 1987). Possibly the effective value of K_a is larger *in situ*, or perhaps most of the acetylglutamate is bound, so that the concentration of the free compound is in the range of K_a (Meijer & Verhoeven, 1984). Whatever the reason for the discrepancy, the linear proportionality shown in Fig. 2 between the concentration of acetylglutamate and the rate of citrulline synthesis is strong evidence for an important regulatory role for acetylglutamate.

Arginine appears to play an important role in the regulation of the syntheses of both acetylglutamate and citrulline. In isolated intact mitochondria, the rate of synthesis of acetylglutamate and of citrulline was half-maximal when the external arginine concentration was between 50 and 100 μM (Fig. 1). This value is similar to that reported by Kawamoto *et al.* (1985) for *N*-acetylglutamate synthase in intact mitochondria. K_a values of 5–10 μM (Shigesada *et al.*, 1978) and about 2.5 μM (Kamemoto & Atkinson, 1985) have been reported for the partially purified enzyme. Freedland *et al.* (1984) showed that mitochondria are able to take up and concentrate arginine. They estimated intramitochondrial arginine concentrations to be 120–300 μM, suggesting that arginine is not important in regulating acetylglutamate synthesis, since the enzyme would appear always to be saturated with modifier. However, *in situ*, *N*-acetylglutamate synthase may actually respond to cytosolic rather than intramitochondrial concentrations of arginine. There is suggestive evidence that *N*-acetylglutamate synthase is associated with the mitochondrial inner membrane and that the activator site for arginine is oriented toward the cytosol (Kawamoto *et al.*, 1985). The observed apparent K_a for arginine, based on the concentration of arginine in the suspending medium (50–100 μM, Fig. 1) is within the reported range of cytosolic arginine concentrations, 30–75 μM (Freedland *et al.*, 1984), and this is consistent with a regulatory role for arginine.

It is noteworthy that the concentration of arginine required to maintain the level of *N*-acetylglutamate found initially in the mitochondria (Fig. 3) is similar to the portal concentration of arginine found when the rats were fed on the corresponding protein diet (Fig. 4). Arginine may be an effective signal whereby high dietary protein increases the liver's capacity to synthesize urea. This assumes that the extrapolation of measured portal concentrations to effects *in vitro* on mitochondria is valid. Arginine affects primarily the $V_{max.}$ of *N*-acetylglutamate synthase, but also decreases the affinity of the enzyme for glutamate (Kamemoto & Atkinson, 1985). Under conditions of high dietary protein intake, both arginine and glutamate are increased, with a resulting increase in *N*-acetylglutamate synthesis.

Stewart & Walser (1980) reported that intraperitoneal injection of very large amounts of amino acids minus arginine caused an increase in liver *N*-acetylglutamate concentration. From that result they concluded that arginine is not important in the regulation of *N*-acetylglutamate synthase. However, the fact that a metabolic change can be observed in the absence of change in the concentration of a putative modifier clearly can provide no information about the changes that would occur when the concentration of that modifier was varied. Our interpretation of their result is that the high concentration of glutamate (40 mM) in the amino acid injection increased *N*-acetylglutamate synthesis by mass action, perhaps over-riding normal control interactions. At more physiologically relevant concentrations of amino acids (30–300 μM; Aikawa *et al.*, 1973), arginine is probably an important regulatory signal.

Together with previously reported observations, our results from assays using isolated mitochondria suggest that arginine, acting through its effect on acetylglutamate synthase, is an important regulator of citrulline synthesis and thus of urea synthesis. This conclusion does not argue for or against a parallel role for glutamate.

This work was supported by National Institute of Diabetes, Digestive, and Kidney Diseases Grants DK 36941 and DK 36967, and by U.S. Public Health Service National Research Service Award GM 07185.

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Received 16 January 1990/13 July 1990; accepted 19 July 1990