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CHANGES IN ISOZYMES OF ADENYLOSUCCINATE SYNTHETASE

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SUMMARY: The activities of the two adenylosuccinate synthetase isozymes in rat liver have been determined under varying dietary conditions. It was found that the expression of the acidic isozyme appears to be coordinated with purine biosynthesis, while the activity of the basic isozyme increases in situations where the animal is metabolizing large amounts of protein. It was found that, under normal conditions, all organs tested contained some of the basic isozyme; it was the only form detectable in skeletal muscle and heart. Liver, kidney, and brain contained both types. In liver the two activities were present in roughly equal amounts, while the acidic form predominates in kidney, brain and spleen.

The function of the purine nucleotide interconversion pathways for IMP and AMP has become the subject of some controversy. The role in the de novo production of AMP is well established, but it has been proposed that these enzymes, adenylosuccinate synthetase and lyase and AMP-deaminase, may operate as a cycle (1,2) which Lowenstein terms the purine nucleotide cycle (PNC). The cycle has been shown to operate in vitro in extracts of rat skeletal muscle (3), liver (4), kidney (5), and brain (6) and demonstrated to operate in vivo in rat brain (7) and rat skeletal muscle (8). Much of the controversy has centered around the proposal by McGivan and Chappell (9) that the PNC is responsible for the production of the ammonia required for urea synthesis. Experiments designed to test this hypothesis have produced conflicting results (10-13) based on apparent insufficient capacity of the cycle to account for urea production especially during starvation.

Within the pathway, the position occupied by adenylosuccinate synthetase is especially interesting; it is a branch point in the pathway of de novo purine synthesis as well as the first enzyme in the reamination half of the PNC. Consis-

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tent with this dual role, Matsuda et al. (14) reported the existence of two distinctly different isozymic forms of adenylosuccinate synthetase present in roughly equal amounts in rat liver. The first, which they designated Type L has a lower K_m for IMP and a higher K_m for aspartate than the second form, designated Type M. Type L was also more strongly inhibited by nucleotides, but less by fructose 1,6-diphosphate. Type M was identical to the enzyme previously purified and characterized from skeletal muscle (15,16). On the basis of these findings, it was proposed that Type L was primarily associated with biosynthesis, while Type M was involved in the PNC (14). In support of the biosynthetic role of Type L, Matsuda, et al. (17) showed that the ratio of the two isozymes changed to about 80% Type L in regenerating rat liver with only a small increase in total activity. Brand and Lowenstein (13) measured the total activity in rat liver before and after starvation, and found no change in total AMP-S synthetase activity, but an 80% decrease in AMP-S lyase activity. Since they also found a greater reduction in the rate of synthesis of AMP from IMP than in the rate of production of IMP, they proposed that the lyase in rate-limiting in starvation. They did not determine the AMP-S synthetase isozyme ratio.

Since the two isozymes of AMP-S synthetase differ so markedly, changes in the relative amounts of the two could drastically affect the regulation of the reaction they catalyze, and therefore the direction of metabolism around that point. Determination of this ratio could be a useful indicator of the relative importance of the biosynthetic and the cyclic aspects of the adenine nucleotide interconversion pathway in different tissues or under different metabolic conditions.

MATERIALS AND METHODS

[U-^{14}C]IMP (42 mCi/mmol) was prepared by enzymatic deamination of [U-^{14}C]AMP (New England Nuclear) using AMP deaminase (Sigma). The IMP was purified by high performance liquid chromatography on a Partisil SAX column (Whatman) using isocratic elution with 5 mM potassium phosphate (pH 3.1). Other chemicals used were reagent quality. For the tissue survey of adenylosuccinate synthetase isozymes, combined extracts of the tissues except muscle were prepared by homogenizing the tissues in 4 volumes of cold 0.25 M sucrose, 30 mM potassium phosphate; 5 mM EDTA, 1 mM dithiothreitol, pH 7.5 (homogenization buffer) using a teflon Potter-Elvehem homogenizer. The homogenates were centrifuged for 1 hr. at 100,000 x g. The supernatants were fractionated with ammonium sulfate and the protein precipitating between 40 and 70% of saturation was used for analysis.

No enzymatic activity was present in the other fractions and the recovery was nearly quantitative. The muscle was homogenized in 6 volumes of the homogenization buffer and stirred for 1 hr. at 4°. The homogenate was centrifuged for 1 hr. at 40,000 x g and the supernatant was fractionated as for the other tissues. The ammonium sulfate precipitates were redissolved in a small amount of homogenization buffer, then desalted on a 28 x 81 mm Bio-Gel P-2 column equilibrated with 10 mM potassium phosphate; 5 mM EDTA, 1 mM dithiothreitol, pH 7.5 (column buffer). The fractions containing ultraviolet absorbing material were pooled and passed through a 14 x 19 mm phosphocellulose column and a 20 x 84 mm DEAE-cellulose column (Whatman DE-52) arranged in series. Non-binding protein was washed out of both columns with column buffer, then the columns were separated and eluted with 1 M KCl in column buffer. The KCl eluates were assayed spectrophotometrically. For the dietary experiments the Sprague-Dawley rats were maintained on Purina chow (Formulab 5008) for at least one week before beginning controlled feedings. At the beginning of the experiment, the rats weighed 170-270 g. Each group was maintained on the experimental diet for 7 days. The chemically defined diets were Purina Basal Diet 5755 and Basal Diet with Hi Protein 5787 and Hi Carbohydrate 5810 modifications. Food and water were available to all groups ad libitum and a normal day-night light cycle was maintained. The rats were killed by decapitation; the livers excised and homogenized separately as described above. An aliquot of the supernatant from each homogenate was taken for assay of adenylosuccinate lyase activity. The 40-70% saturated ammonium sulfate fraction was prepared as before. The precipitated protein from each liver was then dissolved in column buffer and dialyzed against the same buffer to remove the ammonium sulfate. Separate aliquots of each dialyzed protein fraction were passed through 15 x 12 mm phosphocellulose column and 15 x 29 mm DEAE-cellulose column; both equilibrated in column buffer. The columns were washed with column buffer and the combined wash from each was assayed for adenylosuccinate synthetase activity. Total activity for all groups was calculated as the sum of activities washed from the phosphocellulose and DEAE-cellulose columns. The validity of this procedure was checked by washing one column in each group with 1 M KCl. Assays of these washes agreed with assays of non-binding protein fractions for the other column.

Enzyme Assays - Adenylosuccinate synthetase - The spectrophotometric assay mixture contained 20 mM Hepes, pH 7.0, 0.5 mM IMP, 0.1 mM GTP, 5.0 mM aspartate, 10 mM magnesium acetate and 50 μM copper sulfate (to inhibit adenylosuccinate lyase (18)) in a volume of 0.5 ml. The reaction was initiated by the addition of enzyme and was monitored by the increase in A_{280} using a Cary 118 spectrophotometer with the cell compartment thermostatted at 30°. Because of the high absorbance of the assay mixture, 0.2 cm path-length cells were employed. The blank rate was determined by adding 1 mM hadicidin, a specific synthetase inhibitor (20), to the assay. The difference extinction coefficient used was 11.7 x 10^3 $M^{-1}cm^{-1}$. The radioactive assay contained the same components plus 0.1 M potassium acetate, 1 mM phosphoenolpyruvate, 0.5 IU pyruvate kinase, and 9.7 μCi [U-^{14}C]IMP in an assay volume of 0.25 ml. The reaction was initiated by the addition of enzyme and aliquots were removed at 0, 15, 30, 45, and 60 min. The reaction was terminated by immersion in a boiling water bath. The reaction mixture was spotted along with adenylosuccinate, IMP, and AMP standards on prewashed PEI-cellulose TLC plates. The plates with an attached filter paper wick were washed overnight in 50:50 methanol-water, then developed in 0.5 M Na formate, pH 3.4. The reaction was linear for at least 60 min. Blank reaction rate was determined by adding 1 mM hadicidin to the same system.

Adenylosuccinate lyase was measured spectrophotometrically in an assay mixture containing 20 mm Tris-HCl, pH 7.0, 1 mM EDTA and 0.05 mM adenylosuccinate in 1 ml. The reaction was initiated with the addition of enzyme and the decrease in A_{282} was measured (19). The activity was calculated using a difference extinction coefficient of 10.7 x 10^3 $M^{-1}cm^{-1}$.

RESULTS

The L form of adenylosuccinate synthetase has an isoelectric point of 5.9 while the M form has an isoelectric point of 8.9 (14). Therefore, they are easily separable by isoelectric focusing (14,21) and ion-exchange chromatography; the L isozyme binds to DEAE-cellulose (14,17,21) while the M isozyme binds to phosphocellulose (15,21). Analysis of liver extracts by isoelectric focusing indicates that these are the only two observable enzyme species in rat liver (14,21). Therefore, separation of the two forms with small ion-exchange columns provides a simple, rapid method of analysis of the isozyme content of small samples. Since only two forms are present, the amount of the L form in a sample can be taken as the total activity excluded from phosphocellulose (or bound to DEAE-cellulose) and conversely for the M form.

Muscle and heart contain only the Type M form, while kidney and brain contain predominantly the Type L form (82-93% and 84-89% respectively). The two activities are roughly equal in liver. In confirmation of these results, all tissues listed gave a positive precipitation reaction in Ouchterlony double immunodiffusion tests with antiserum against the muscle isozyme. Extracts of spleen and lung also gave positive reactions. Moreover, analysis of spleen indicates that the activity is almost entirely L form (>95%).

The changes in the activities of rat liver AMP-S synthetase isozymes in response to various dietary regimens are shown in Table I. In rats fed the purine-free basal diet, Type L predominates, while increasing the carbohydrate content of the diet shifts the ratio back near control levels. Feeding the high protein diet shifts the ratio still further, to essentially equal activity, and with starvation the Type M activity predominates. Total activities of AMP-S synthetase and AMP-S lyase are also shown in Table I. No significant change in the total activity of either enzyme was found except possible increases on high protein and high carbohydrate diets.

DISCUSSION

This study clearly shows that the levels of the two AMP-S synthetase isozymes in liver are sensitive to the nutritional state of the animal. In situations where

Table I
Effect of Diet on Liver AMP-S Synthetase Isozyme and AMP-S Lyase Levels

Diet	AMP-S Lyase[a] nm/min/g wet wt.	AMP-S Synthetase[a] nm/min/g wet wt.	% L[b] form of AMP-S Synthetase
Chow	231.0 ± 12.0	78.8 ± 3.1	59.3 ± 0.9
Purine-free Basal	233.2 ± 4.6 NSD	81.8 ± 3.5 NSD	71.0 ± 1.1 p<0.001
Purine-free Hi Carbohydrate	268.2 ± 9.0 p<0.05	86.0 ± 5.3 NSD	57.3 ± 2.9 NSD
Purine-free Hi Protein	269.2 ± 10.8 p<0.05	95.4 ± 6.0 NSD	49.5 ± 1.9 p<0.001
Starvation - 7 days	205.2 ± 28.6 NSD	84.5 ± 2.3 NSD	33.8 ± 3.1 p<0.001

[a] Each group value is the mean ± S.E.M. for six rats. The Student t test was used to determine the significance of the change observed. The other diets are compared to Chow group. NSD is not statistically different (p>0.05).

[b] The percent of the total activity as the L isozyme.

increased biosynthesis would be required, i.e. a purine-free diet, the activity of the L isozyme increases. The decrease in L activity with respect to the basal diet seen in the high carbohydrate and high protein groups is possibly due to increased availability of substrates for purine de novo synthesis. The decrease in L activity in starvation further indicates that this isozyme may be regulated for biosynthesis.

The shift toward the M isozyme with high protein diet and starvation, conditions which force the animal to derive most of its energy from protein, supports the proposal of Matsuda, et al. (14) that it is involved in the deamination of amino acids through the purine nucleotide cycle. Matsuda, et al. (14) showed that the AMP-S synthetase Type M isozyme has a lower K_m for aspartate, is more sensitive to inhibition by fructose 1,6-diphosphate, and less sensitive to inhibition by nucleotides than Type L. These properties indicate that the M isozyme could be regulated coordinately with glycolysis (or gluconeogenesis) as proposed by Tornheim and

Lowenstein (22) for the operation of the PNC in skeletal muscle. The enzyme could also be effected by the availability of aspartate as was found by Henderson, et al. (23) in Ehrlich ascites cells. The increase in Type M activity under conditions used in this study where the animal must rely on protein for most of its energy is consistent with the idea that it is involved in the PNC.

The lack of change in AMP-S lyase activity, even in starvation, is contradictory to the results obtained by Brand and Lowenstein (13), who found that the activity of the lyase dropped 89% after 4 days of starvation. The rats used in their study averaged 165 g initially and lost over 50% of their body weight in 4 days. The rats employed in this study averaged 276 g and lost only 30% in 7 days. These larger rats may be more resistant to starvation and exhibit different changes in metabolism. Additionally, as the duration of starvation increases, the lyase level may rise again as the animal becomes increasingly dependent on muscle protein. Brand and Lowenstein (13) also showed that the rate of synthesis of AMP from IMP decreased more than the rate of production of IMP. Since they found no change in total AMP-S synthetase activity, they proposed that the lyase is rate-limiting in starvation. However, the results reported here indicate that simple measurement of the total synthetase activity may not be valid since the isozyme ratio and therefore the regulation of the reaction is altered drastically with no apparent change in total activity. Since the M synthetase has a 6-fold higher K_m for IMP than L (13,24) (30 μM for L and 200 μM for M) its predominance in in starvation could also account for the reduction in the rate of conversion of IMP to AMP.

In light of the results obtained on the influence of diet on the liver AMP-S synthetase isozyme ratio, the tissue distribution of the isozymes could possibly give some indication of the capacity to produce AMP or the feasibility of the PNC in the various organs. Of the organs tested, liver, kidney, spleen and brain appear to possess a significant capacity for biosynthesis, while heart and skeletal muscle may not. However, the actual situation is probably not that clear cut; there is no reason to assume that the two functions are strictly divided between the two isozymes. For instance, the operation of the PNC has been dem-

onstrated in brain (6) as well as in skeletal muscle (8) and in cell extracts of liver (4) and kidney (5) while Sheehan et al. (25) found that ^{14}C-formate was incorporated into purines by intact rat skeletal muscle. Also, since kidney and brain contain several different cell types, interpretation of the data for these two organs is further complicated. The implications of this phenomenon with respect to cell function are unclear. However, the findings presented here indicate that the isozymes of AMP-S synthetase are important for the regulation of metabolism under a variety of conditions.

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