Published in final edited form as:

Biosci Rep. 2008 October ; 28(5): 239–249. doi:10.1042/BSR20080090.

THE MITOCHONDRIAL POOL OF FREE AMINO ACIDS REFLECTS THE COMPOSITION OF MITOCHONDRIAL-DNA ENCODED PROTEINS:

INDICATION FOR A POST-TRANSLATIONAL QUALITY CONTROL FOR PROTEIN SYNTHESIS

Catherine Ross-Inta, Chern-Yi Tsai, and Cecilia Giulivi*

Department of Molecular Biosciences, School of Veterinary Medicine, University of California
Davis, CA 95616

Abstract

Mitochondria can synthesize a limited number of proteins encoded by mtDNA by using its own biosynthetic machinery, whereas most of the proteins are imported from the cytosol. It could be hypothesized that the mitochondrial pool of amino acids follows the frequency of amino acids in mtDNA-encoded proteins or alternatively, the profile is the result of the participation of amino acids in pathways other than protein synthesis (e.g. heme biosynthesis, aminotransferase reactions). These hypotheses were tested by evaluating the pool of free amino acids and derivatives in highly-coupled, purified liver mitochondria obtained from rats fed a nutritionally adequate diet for growth. Our results indicated that the pool mainly reflects the amino acid composition of mtDNA-encoded proteins suggesting that there is a posttranslational control of protein synthesis. This conclusion was supported by the following findings: one, correlation between the concentration of free amino acids in the matrix and the frequency of abundance of amino acids in mtDNA-encoded proteins; two, the similar ratios of essential-to-nonessential amino acids in mtDNA-encoded proteins and mitochondrial pool of amino acids; three, lack of a correlation between codon usage or tRNA levels and amino acid concentrations. Quantitative information on the mammalian mitochondrial content of amino acids, such as that presented in this report, along with functional studies will help us better understand the pathogenesis of mitochondrial diseases or the biochemical implications in mitochondrial metabolism.

Keywords

mitochondria; protein synthesis; amino acids; essential amino acids; nonessential amino acids; liver; nutrition; diet

INTRODUCTION

The mitochondrial DNA encodes for 13 polypeptides, namely 7 subunits of Complex I (ND1–ND6 including ND4L), cytochrome *b*, 3 subunits of Complex IV (COX I–III), and 2

*To whom all correspondence should be addressed: Dr Cecilia Giulivi, University of California Davis, 1120 Haring Hall, One Shields Ave., Davis, CA 95616.

subunits of Complex V (ATPase 6 and 8; [1]), 2 rRNAs, and 22 tRNAs. It can synthesize a few, but critical proteins by using its own biosynthetic machinery, whereas most of the proteins are imported from the cytosol. It could be hypothesized that the mitochondrial pool of amino acids is result of protein synthesis of mtDNA-encoded proteins and degradation of mitochondrial proteins (regardless of the genome origin), in addition to those amino acids whose pathways are involved in others not related directly to protein synthesis (e.g. heme biosynthesis, aminotransferase reactions). Alternatively, the mitochondrial pool of free amino acids might be more influenced by one of these pathways in particular (e.g. following the profile of amino acids in mtDNA-encoded proteins). To test these hypotheses, reliable data on the pool of free amino acids in mitochondria should be available. However, very few publications and most of them using the technology available in the 60s–70s have been presented [2–4]. Thus, the main goal of this study was to provide a quantification of free amino acids and derivatives in highly-coupled, purified liver mitochondria obtained from rats fed a nutritionally adequate diet for growth. Furthermore, the putative differences between the cytosolic and mitochondrial pools of free amino acids were evaluated to ascertain if these are different pools to support different functions or if the mitochondrial pool is similar to the cytosolic one. In this regard, the occurrence of correlations between the levels of mitochondrial free amino acids and those of tRNA, amino acid content in mitochondrial polypeptides, or codon usage were also tested. Finally, comparison between the mitochondrial, cytosolic, and serum pool of amino acids were evaluated to ascertain similarities and differences of these pools.

Quantitative information on the mammalian mitochondrial content of amino acids, such as that presented in this report, along with functional studies will help us better understand the pathogenesis of mitochondrial diseases or the biochemical implications in mitochondrial metabolism when protein deficiencies are encountered in diets.

EXPERIMENTAL

Chemicals and biochemicals

EDTA, EGTA, sodium succinate, mannitol, sucrose, HEPES, bovine serum albumin (fatty-acid free) were all purchased from Sigma (St. Louis, MO). Tris, glycine, sodium chloride, and potassium chloride were purchased from Fisher (Pittsburg, PA). All other reagents were of analytical grade.

Animals and diet

The experiments described below were approved by the UC Davis Animal Care and Use Committee. Male Sprague-Dawley rats (6-wks old) weighing 180–200 g were purchased from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN, except as noted). All cages were kept in a vivarium maintained at 22 ± 1 °C, with a 12-h light:dark cycle, following NIH guidelines for animal care and use. The lights were set to go out at 19:00 h. Cage maintenance was conducted during the light cycle.

Briefly, after arrival in our laboratory, rats were fed an amino-acid based diet. The diet was prepared in our laboratory using purified ingredients and free L-amino acids. The diet was

amino acid-based with a full complement of all essential amino acids at ratios based on the egg protein (S1 Tables Ia–Ic). Over the testing period, the rats had free access to their food 24 h/d, except when cage maintenance was conducted. At day 9, the rats were removed from their cages and taken to the surgery room, where they were euthanized with CO₂. Rats were euthanized between 9–10 am. The livers were quickly removed from the rats, wet weights recorded, and rapidly homogenized for mitochondria isolation or amino acid analyses.

Mitochondria isolation

Rat liver mitochondria were isolated as previously described by our lab [5, 6]. Briefly, purified mitochondria from homogenized livers or hearts were collected by differential centrifugation followed by Percoll gradient and multiple washes in MSHE buffer (220 mM D-Mannitol, 70 mM sucrose, 0.5 mM EGTA, 2 mM HEPES, 0.1% fatty-acid free bovine serum albumin) and 150 mM KCl. A faster procedure was designed based on that published by [7]. Essentially, 0.2 to 0.4 g of liver were homogenized in cold MSHE, diluted with an equal volume of 36% Percoll in MSHE, and applied directly on a Percoll gradient over two preformed layers containing 36% and 55% Percoll. The gradients were centrifuged at 30,700 g for 5 min. The third fraction from the top was collected, resuspended in MSHE, centrifuged at 7,300 g for 5 min, and finally resuspended in 150 mM KCl.

Oxygen Consumption

The oxygen uptake of mitochondria was measured using a Clark-type O₂ electrode from Hansatech (King's Lynn, UK) at 30°C [5, 6]. Intact, purified mitochondria were tested in 1 ml of reaction buffer containing 0.225 M sucrose, 5 mM MgCl₂, 20 mM KCl, 10 mM potassium phosphate, and 20 mM Hepes/KOH, pH 7.4 in the presence of 1 mM malate–10 mM glutamate (malateglutamate) or 10 mM succinate as substrates and 0.45 mM ADP. The P/O ratios were determined measuring the increase in the uptake of oxygen caused by the addition of a known amount of ADP, as described by Chance and Williams [8].

Amino acid analyses

Tissue samples were immediately homogenized in ice-cold 3% sulfosalicylic acid (10 ml: 1 g of liver wet weight; 10 ml: 1 g of mitochondrial protein; 1 V/1 V of serum to SSA) spiked with 2.45 μM L-g-amino-*n*-butyric acid as the internal standard. Each sample was then centrifuged at 14,000 g for 30 min at 4°C. All samples were processed for amino acid analysis using a commercially available kit [AccQ Tag, Waters [9, 10]]. Amino acids were visualized using HPLC with fluorescence detection.

Statistical analyses

Data were analyzed using the ANOVA test, and results were expressed as means SEM except where noted.

RESULTS & DISCUSSION

Free amino acids in rat liver mitochondria

Male, 4-wks old Sprague-Dawley rats were fed with an amino-acid based diet containing all nutrients for adequate growth (diet with 0.87% Thr as described in [11]). To determine the amino acid levels present in liver mitochondria, and considering that these concentrations fluctuate significantly after or before a meal [12, 13], it is important to indicate that the rats used in this study were euthanized between 9 and 10 am, about 2–3 hours after their first meal, thus, the amino acid pool reported in this study should be taken as representative of a postprandial period. Percoll-purified, liver mitochondria showed negligible levels of contamination with other subcellular compartments and high coupling of the electron transport to oxidative phosphorylation [6]. Intactness and functionality of these mitochondria were assessed by evaluating the respiratory control ratio (RCR) and P/O numbers. The RCR with malateglutamate was 10.7 ± 0.1 and with succinate 6.6 ± 0.2 . The P/O numbers with each substrate were 3.2 ± 0.2 and 2.29 ± 0.04 indicating that the 3 phosphorylation sites were operational.

The concentrations of free amino acids and derivatives found in these mitochondria are shown in Table I. The total amount of amino acids found in this study was, in general, within values reported before for various species and organs (S1). The relative high concentration of amino acids reported in this (about 35 mM) and other studies (S1) had raised the possibility that they might contribute significantly to the osmolarity of these organelles, whereas that of either serum (or plasma) or cytosol is constituted mainly by inorganic electrolytes. However, if the mitochondrial osmolarity has been calculated as 300-to-330 mM where the sum of the concentrations of potassium, sodium, magnesium, chloride, orthophosphate, and organic phosphate is between 150 and 180 mM, and the internal concentration of sucrose is about 150 mM (when isolating rat liver mitochondria with 0.25 M sucrose; [14, 15], then the highest concentration of osmotically active amino acids would be 150 to 180 mM, about 20% of the observed experimental concentrations. The relative high concentration of amino acids might also explain the low rate of radioactive label incorporation into mitochondrial proteins if cells had not been previously starved in Met-free medium for an adequate period [16] because, as suggested by [4], the relative large size of the amino acid pool leads to dilution of the isotopically labeled amino acids, resulting in an underestimated amount of incorporated radioactivity.

When the amino acid profile for rat liver mitochondria was compared to others published before (S1), the main difference was that, to our knowledge, this is the first study that reports values for Asn, Cys, Pro, Trp, and Tau, whereas these compounds were not detected before because of either the method used (e.g., Asn detected as Asp) or the concentrations were below the detection limit [2–4]. In addition, we evaluated several other biologically relevant compounds, whose concentrations were not sought (e.g., anserine, carnosine, 1- and 3-methyl-His).

The amounts of essential (EAA) and nonessential (NEAA) amino acids in rat liver mitochondria were 159.4 and 135.7 nmol/mg protein (Table II) resulting in an EAA:NEAA ratio of 1.18. This ratio was distinct from that obtained for rat liver (0.44) and serum (0.57),

suggesting that these pools are highly compartmentalized, and that a gradient of EAA exists from blood → mitochondria. The experimental ratio of EAA:NEAA of rat liver mitochondria (1.18) was similar to that calculated from Baird ([2]; 1.3), but quite different from those calculated from others (0.2 to 0.6, [3, 4]). In fact, these latter ratios were more similar to rat liver EAA:NEAA ratios obtained by us (0.44) and others (0.12 [17, 18], 0.2 [19]), but below the values found for serum or plasma (0.57, this study; 0.3–0.5 [20]; 0.65 [18]; 0.7 ([19]; 0.93 [17])). This indicated that, in these reports (S1, [4]), a crosscontamination between mitochondria and cytosol had occurred during the isolation procedure.

If outlier values are excluded (2-times above or half of our values; underlined numbers in Table II S1), our numbers were more similar to those reported by Baird [2]. However, this last report failed to measure concentrations for Arg, Asn, Cys, Gln, Pro, Trp, Orn and Tau, and relatively high (Glu, Lys, and Ser) or low (Met and Tyr) concentrations for other amino acids.

Although the EAA:NEAA calculated from [3] seemed to be closer to our value, a closer inspection of the distribution of sulfur-containing compounds indicated that most of Cys and GSH were recovered as cystine, cysteic acid, and GSSG. This is indicative of samples being exposed to oxidative stress. If this were the case, then the permeability of the mitochondrial membranes would be compromised resulting in significant changes in metabolite concentrations. In contrast, almost 60% of the low-molecular weight sulfur-containing compounds (GSH, GSSG, Cys, Met, and Tau) in our study was comprised by glutathionyl equivalents. The high ratio of [GSH]/[GSSG] found in this study was comparable to data obtained before by us [21] and others [22, 23], indicating that these mitochondria were minimally exposed to oxidative stress.

Other significant reasons for the differences between our and other studies could be related to model-specific differences (sex, age, strain; [24–26]), and time of sampling (circadian fluctuations of amino acids; [27]).

It could be argued that the mitochondrial pool reported in this study is the result of mitochondrial proteolysis that might have occurred during the isolation procedure. Several reasons are against this possibility: one, throughout the entire isolation procedure, EDTA and EGTA, metalloprotease inhibitors, were present at concentrations relevant to exhibit this activity. When in addition to these, other proteolytic inhibitors ([4-(2-Aminoethyl)benzenesulfonylfluoride, aprotinin, bestatin, E-64, phenylmethylsulfonyl fluoride, and pepstatin A) were present throughout all steps of mitochondria isolation and purification, no changes in the quality and quantity of amino acids from mitochondria was obtained. Two, the isolation procedure was performed at 0–4°C (on ice, buffers, glassware, rotors, refrigerated centrifuge, etc.) to minimize enzymatic activities, and the procedure was performed within 2 h to minimize the time of the handling of samples and their storage on ice. When a different procedure to isolate mitochondria was performed that allowed to obtain mitochondria in less than 45 min, no significant changes in the amino acid profile was obtained. Even if any unavoidable proteolysis or leakage were to occur during our procedure, it has been shown that incubating mitochondria (not purified and without

proteolytic inhibitors) at 0°C for 4 h results in a total amino acid increase of 2-to-6 mM [28]. These increases are within the experimental error, and their contribution could be considered negligible to the pool of free amino acids. Three, throughout the procedure, isolation buffers were supplemented with 0.1% fatty-acid free bovine serum albumin. This protein, not only binds fatty acids which may uncouple mitochondria, but also at this concentration provided an alternative substrate for putative proteases, sparing mitochondrial proteins from degradation. Finally, if the amino acids present in the matrix were the result of proteolysis during the isolation procedure, then the ratio of EAA:NEAA should be similar to that of the most abundant mitochondrial proteins (e.g., carbamoylphosphate synthetase I, glutamate dehydrogenase, α - and β -subunits of ATPase). These ratios are (in average) 0.79 significantly different from the one found for the mitochondrial pool (1.18) indicating that the values for these amino acids reflect their steady-state concentrations under the experimental conditions used in this study.

Comparison between serum, liver, and mitochondrial pools of free amino acids

It has been shown that some metabolites follow a non-uniform distribution between the mitochondrial and cytosolic compartments (e.g., [ATP+ADP+AMP] 13 mM vs. 8 mM; [29]) whereas others have similar concentrations (GSH, 7 to 10 mM; [21, 23]). Following this reasoning and considering several assumptions (see under Table II), the mitochondrial concentrations of the amino acids in the mitochondrial matrix water were calculated. We tested whether the mitochondrial pool of free amino acids followed that in the cytosol or serum, or if they constituted an independent, different pool. To this end, the serum and liver concentrations of free amino acids were evaluated in parallel to those in mitochondria (Table II). Most amino acid and derivative concentrations were higher in mitochondria than in the cytosol or serum, with a few exceptions: citrulline, glutamic acid, hydroxyPro, 1-methyl-histidine, proline, sarcosine, and Tau. Even if the amino acids are classified into essentials and nonessentials, still mitochondria have the highest concentration of both, and exhibited the highest ratio of EAA:NEAA (Table II). However, considering the low K_m of all amino acids for their respective aminoacyl-tRNA synthetases (nM to low μ M; [30, 31]), the amount of tRNAs (μ M; [32]) and amino acids (mM) in each compartment (S1), no significant changes in the acylation of tRNAs would be expected in either compartment, resulting in a favored protein synthesis as it would be expected for a growing, well-fed animal. Moreover, given that codon misreading during amino acid starvation presumably comes about because the amount of cognate charged tRNA drops to a very low level increasing the likelihood of misreading or misincorporation by a nearly cognate tRNA [33], then the high levels of mitochondrial (and cytosolic) amino acids might also prevent drops in tRNAs, preventing misreading frequency and assuring fidelity of protein synthesis.

Role of amino acids in mitochondria

The pool of free amino acids in mitochondria could be considered to represent the resultant of the difference between the input (represented by the influx from the pool present in the liver cytosol, amino acids from mitochondrial protein degradation, and amino acids formed by other processes) and the output (amino acids used for mitochondrial protein synthesis and other processes). If it is assumed that in a well-fed, young and growing animal, the rate of synthesis will be favored over that of proteolysis, the contribution of mitochondrial protein

degradation to the pool of mitochondrial free amino acids will be considered negligible. Thus, if the “original” pool of mitochondrial amino acids is considered identical to that of liver cytosol, and from this, free amino acids (determined in this study) and amino acids used for mitochondrial protein synthesis are subtracted, it would be possible to evaluate the distribution and use of each amino acid in terms of functions other than protein synthesis (e.g., transamination reactions). Assuming that the main factor that determines the different molar abundance of mitochondrial Complexes is the rate of synthesis of their subunits [34], using the relative abundance of different Complexes from beef heart mitochondria [35], the primary sequences of rat mtDNA-encoded subunits (from Expasy web site), and setting as an upper limit the total amino acid concentration, the distribution of each amino acid was estimated (Fig. 1). In almost half of the cases (His, Ile, Leu, Met, Thr, Trp, Asn, Cys, Tyr), the addition of the concentration of amino acid used for mitochondrial protein synthesis and that of the free amino acid accounted for the total amino acid concentration, leaving negligible amounts for other functions. Conversely, amino acids involved in transamination/nitrogen metabolism (Ala, Asp, Gln, Glu, Gly), presented a higher concentration destined to other functions than protein synthesis. In this category, amino acids whose catabolic pathway is mainly mitochondrial (Lys, Thr, and Gly; indirectly Ser → Gly) were also included.

If the main role of some matrix free amino acids were to support the synthesis of mtDNA-encoded proteins, then excluding those that have a main role in functions other than protein synthesis, a correlation between their levels and the amino acid abundance in mtDNA-encoded proteins could be expected. The putative correlation between all free amino acid in the matrix and the amino acid content in mtDNA-encoded proteins is shown in Fig. 2. A positive slope was found indicating that there was a trend between these variables (Fig. 2); analysis of the regression line indicated a correlation (r^2) of 0.54 ($n = 20$; Pearson's analyses $p < 0.05$). When amino acids known to be catabolyzed predominantly in rodent mitochondria and those involved mainly in functions other than protein synthesis, were excluded from the analysis, the correlation coefficient was $r^2 = 0.93$ ($n = 10$; Pearson's analysis indicated a $p < 0.05$), indicating a high correlation.

No amino acid profile of a single polypeptide encoded by mtDNA was specifically more correlated to the amino acid pool; instead, all polypeptides (except ATP8 with $r^2 = 0.58$) had correlation coefficients between 0.8 and 0.9.

It could be argued that amino acids that have other function besides being substrates for protein synthesis might be at limiting concentrations, even if their concentrations are not among the lowest ones. However, several examples demonstrate that the mitochondrial concentrations are not limiting for protein synthesis (see above) or other functions. For example, Arg concentrations were 30-times higher than the K_m for Arg of nitric-oxide synthase [5], confirming as we indicated before [5, 36], that Arg concentrations allow full saturation of this enzyme, and about 400-times higher than that required for *N*-acetylglutamate synthase (NAGS) activation [37]. In another example, Gly concentrations would allow a 6% of the maximal activity of 5-amino-delta-levulinate synthase (K_m for Gly = 23 mM; [38]) the first enzyme in the heme biosynthetic pathway, assuming a Michaelis-Menten behavior and full saturation with the other substrate, succinyl-CoA. It has been

reported that glutamate dehydrogenase appears to be saturated at intramitochondrial concentrations of approximately 3.7 nmol Glu/mg protein [39, 40]. At the mitochondrial concentrations of Glu, it would be expected that this enzyme would be operating at only 10% of its maximal activity, whereas a higher activity would be expected when an influx of Gln or Glu is directed to liver (e.g., early starvation).

Taurine, Val, and Lys

Although the precise role for Tau is still not fully understood, it is known that it is not present in mtDNA-encoded proteins. Given that its free concentration is relatively small, a role for osmolarity control [41–43], as it has been proposed for this compound, seems unlikely (Table I). Thus, it seems that taurine is fulfilling another role in mitochondria. In this regard, a link between Tau deficiency and cardiomyopathies in cats has been established [44], and more recently, it has been found that Tau is present in modified uridines at the wobble position of mitochondrial tRNAs, namely 5-taurinomethyluridine in tRNA^{Leu} and 5-taurinomethyl-2-thiouridine in tRNA^{Lys} [45, 46]. The Tau wobble modification-deficiency has been shown to result in a translational defect, that is likely to contribute significantly to mitochondrial dysfunction associated with mutations in mt-tRNA^{Lys} or mt-tRNA^{Leu} (as in the case of MERFF and MELAS).

An unexpected result was obtained with Val. Although Val is quite abundant in mtDNA-encoded proteins, the concentration destined for other functions was higher than that estimated for protein abundance. This finding is in contrast with that found for the other BCAA (Ile and Leu), whose concentrations were mainly used to sustain mitochondrial proteins. This can be explained by the relatively lower (2-times lower than those for the other two BCAA; recalculated from [47]) second-order rate constant for Val catabolism by the BCAA metabolome.

One of the most abundant amino acids in mitochondria was found to be Lys. The concentration of Lys was not only higher than that required to support protein synthesis, but also higher than for its catabolism. In rodents, Lys is predominantly catabolyzed in liver mitochondria by the subsequent action of two enzymes, Lys- α -ketoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH) [48, 49]. The concentration of Lys was 2-times the K_m for purified LOR (K_m for Lys = 1.5 mM; [50–52]), indicating that its catabolism is not limited by Lys concentrations. It could also be inferred that the uptake of Lys is not the rate-limiting step for Lys oxidation, in contrast with [27] using rats fed various casein diets. The LOR and SDH activities contribute not only to the general nitrogen balance in the organism (amino acid catabolism), but also to the controlled conversion of Lys into ketone bodies [52–55]. These energetic compounds can be used as substrates for energy in situations of limited carbon supply. It has been found that the LOR and SDH activities increase in starved mice in comparison with well-fed animals [56]. Therefore, under our experimental conditions, i.e. well fed animal, it is expected that the ketogenesis pathway would be depressed, and as a consequence, the use of Lys for such purpose would also be limited by other regulatory pathways, leading to higher steady-state concentrations of this amino acid. Two facts support this hypothesis: Lys (along with Tyr and Phe) plays a major role in hepatic ketogenesis [57]. Two, the catabolism of Lys in a well-fed animal (without

the need for an active ketogenesis) could be regulated at the level of LOR and SDH concentrations (long-term regulation) or limiting the substrate for these enzymes (short-term regulation). Given that Lys and probably NADPH concentrations are higher than the K_m required for full LOR activity [50–52], it could be proposed that the availability of α -ketoglutarate required for this catabolic pathway is compromised given that its K_m (1 mM; [50–52]) is 3-orders of magnitude higher than its calculated mitochondrial concentration from the glutamate-oxaloacetate transaminase reaction ($1.4 \mu\text{M}^1$; [40, 58]. Assuming Michaelis-Menten kinetics for LOR, the LOR activity would be $<0.1\%$ of its $V_{\max}$ even if all α -ketoglutarate would be available for this reaction.

Urea cycle activity: Ornithine and citrulline

The concentration of Orn was in the intermediate range of most amino acids, whereas that of citrulline was undetectable. This might indicate that the synthesis of citrulline from Orn at the level of Orn transcarbamoylase was depressed. Since this enzyme utilizes Orn and carbamoyl phosphate as substrates for the production of citrulline, it could be assumed that carbamoyl phosphate synthetase (CPS I) was not activated under these conditions. CPS I catalyzes the first committed step in the urea cycle and is allosterically activated by *N*-acetyl-glutamate (NAG). NAG is synthesized from Glu and AcetylCoA by NAG synthase (NAGS). When amino acid breakdown increases, the concentration of Glu increases as a result of transamination. Glu stimulates the synthesis of NAG. The resulting activation of CPS I increases the rate of urea production, facilitating the disposal of nitrogen as urea. Considering that the flux of Glu is primarily committed to the glutamate dehydrogenase and glutamate-oxaloacetate transaminase reactions, the amount of Glu available to NAGS was probably lower than the K_m of Glu for NAGS (3.7 mM; [37]). This situation suggested that there is a limited amount of amino acid breakdown, situation expected for a growing, well-fed animal.

Lack of correlation between tRNA concentrations, codon usage, and mRNA half-lives with free amino acids in mitochondria

A positive correlation has been found between tRNA in reticulocytes and the abundance of their respective amino acids in rabbit hemoglobin [59, 60] suggesting that the tRNA content of reticulocytes is specialized for hemoglobin synthesis. In addition, Kurland and collaborators have shown that tRNA abundance can be correlated with the values of codon frequencies [61–63]. Following this reasoning, we wanted to ascertain if there was a correlation between the levels of tRNA in mitochondria and the abundance of the respective amino acids in the mitochondrial matrix. We used the concentrations of tRNA from HeLa mitochondria [32] because the relative steady-state levels of the tRNAs examined in various species have rarely been found to vary more than 10-fold, and for the majority of the tRNAs, the variation was less than 3-fold [64–67]. No correlation was found between tRNA levels and the concentrations of free amino acids in mitochondria ($S1$; $r^2 = 0.003$). Even if tRNA^{Val} and tRNA^{Phe} , the most abundant tRNAs, were excluded, the correlation did not improve significantly, indicating that the levels of tRNAs did not change with the concentrations of amino acids. Indeed, the concentrations of free amino acids were (in average) 30,000-fold

¹Glutamate-oxaloacetate $K_{eq} = 6.61$; Glu and Asp concentrations from Table I; oxaloacetate concentrations from [58].

than those of tRNAs. This situation would result in full saturation of tRNAs with the corresponding amino acids, optimizing the rate of protein synthesis. Furthermore, considering that severe imbalance of tRNAs can lead to alterations of translation, such as ribosomal frameshifting, amino acid misincorporation, and suppression of termination codons [33, 68, 69], the ratio of amino acids to tRNAs seem to be adjusted to optimize the accuracy of mitochondrial translation.

We also examined whether a correlation occurred between the concentration of free amino acids and the codon usage or the mRNA half-lives of mitochondrially-translated proteins (taken from [70]). For the data set shown, there was not an evidence of a significant regression line (S1; $r^2 = 0.02$).

Lack of correlation between MesoH, GRAVY, and H17 with free amino acids in mitochondria

It has been proposed that the absence of editing motifs in mitochondrial Phe and Leu aminoacyl-tRNA synthetases (mt-aaRSs) leads to a lower fidelity during aminoacyl-tRNA synthesis in this compartment than in the cytosol [71]. The loss of the editing domain from mammalian mitochondrial Leu- and Phe-RSs is in sharp contrast to the general pattern of synthetase evolution, where modules with new functions have been systematically integrated into existing proteins [72]. (In addition, challenges the hypothesis of the origin of Eukaryotes by the symbioses of two partners of well-defined groups, such as for example, *Archea* with α -*Proteobacteria* (see [72] for a complete discussion)). This critical difference between mitochondrial and cytosolic aaRS evolution and function indicates that mitochondrial protein synthesis quality control might be focused at a co- or post-translational step because the translation in this compartment could be inherently less accurate. Given the small number of proteins synthesized by mitochondria, reduced accuracy might be overcome by the existence of an efficient proteolytic system. Proteins accurately translated and folded would reach the inner membrane to become functional, whereas the rest would be degraded. In this regard, non-assembled cytochrome oxidase subunit 2 from yeast is a substrate for the *i*-AAA protease [73, 74]. If this were the case, then mtDNA-encoded proteins rich in Phe and Leu, or as an extension, in hydrophobic amino acids, would be more prone to proteolysis and the matrix pool of amino acids should essentially reflect the composition of these mtDNA-encoded proteins according to their hydrophobic character. To test this hypothesis, we approached the problem using three different variables to assess hydrophobic content: one, GRAVY or grand average of hydropathicity; two, MesoH or mesohydrophobicity, which is the average regional hydrophobicity over an extended region of amino acids, in this case, 100 amino acids; and three, H17, calculated as the maximum local hydrophobicity of a segment with a scanning window length of 17 amino acids that possess the highest mean hydrophobicity per residue. From each individual plot of free amino acids and amino acid composition for each mtDNA-encoded subunit, a correlation coefficient was obtained. These coefficients from each mtDNA-encoded subunit were plotted against GRAVY, MesoH, and H17. None of these parameters showed a statistical significance with r^2 of 0.001, 0.002, and 0.54 for GRAVY, MesoH, and H17, respectively. Thus, these results indicated that hydrophobic proteins (evaluated by using these three different parameters) were not necessarily degraded at a higher rate than others. In

agreement with this conclusion, it has been reported that about 10%-to-15% (without ATP) and 40% (with ATP) of newly polypeptides synthesized by isolated rat liver mitochondria are degraded within an hour of incubation [75], and that no single polypeptide is specifically degraded; instead all mtDNA-encoded proteins disappeared to a similar extent, in agreement with our conclusions.

Conclusions

The pool of free amino acids present in the mitochondrial matrix seems to be the result of proteolytic degradation of mtDNA-encoded proteins, rather than fulfilling an amino acid profile to suit mitochondrial protein synthesis. This was inferred from the correlation between the amino acid concentrations in the matrix and the amino acid abundance in mtDNA-encoded proteins, the relatively high ratio of amino acids to RNA concentrations when compared to those in the cytosol, and the similar EAA:NEAA ratio between the matrix pool of amino acids and the amino acid composition of mtDNA-encoded proteins. No correlation was found with tRNA levels or codon usage for protein synthesis, as determined from the DNA sequence and the rates of synthesis of the various polypeptides. The mitochondrial pool of amino acids appeared distinct from that in the cytosol and in serum (individual values and EAA:NEAA), reinforcing the concept that this pool is kept separate from those in the cytosol of liver and plasma (or serum) fulfilling a different role. Indeed, the EAA:NEAA ratio obtained with mitochondria (1.18) resembles more that found in rat red blood cells (0.8; [76]), cells that before losing all organelles, synthesized large quantities of a few proteins (globins).

If the main function of the amino acid pool present in the mitochondrial matrix were to sustain mitochondrial protein synthesis, and considering that all tRNAs would be saturated at the matrix concentrations of amino acids, then why the profile of several free amino acids in mitochondria is similar to that of the mtDNA-encoded proteins? It could be argued that the matrix pool reflects mainly either the catabolism of oxidatively modified mitochondrial proteins or that of the most abundant ones. There are two main reasons that do not support these options. One, given that mitochondrial proteins are exposed to higher oxidative and nitrative stress because these organelles constitute the main source of intracellular reactive oxygen and nitrogen species, and that mitochondrial nitrated proteins have a significant faster turnover than the native, unmodified ones [6], then the matrix pool should reflect the composition of the most nitrated proteins, not necessarily the ones encoded by mtDNA. The EAA:NEAA ratio of the most nitrated proteins extracted from our work [6] was calculated between 0.6-to-0.8, significantly lower than that observed for the mitochondrial amino acid pool. Two, the ratio of EAA:NEAA in the matrix did not reflect that from the most abundant proteins (e.g., carbamoylphosphate synthetase I, glutamate dehydrogenase, α - and β -subunits of ATPase) indicating that the mitochondrial pool is not the main result of the catabolism of most abundant proteins.

An alternative scenario could be envisioned if the translation of mitochondrial proteins were less accurate than the cytosolic one, their critical difference being their different origin in evolution (ancestral partners in endosymbiosis plus exchange of genetic material with others). Studies focused at understanding aaRS evolution indicate that mitochondrial protein

synthesis quality control could be focused at co-translational or post-translational steps: a misfolded protein is rapidly degraded by the mitochondrial proteolytic system because it is either not recognized by the corresponding chaperone (e.g., Oxa1) or by other subunits within a given Complex (e.g., cytochrome *c* oxidase subunits). Given the small number of proteins synthesized by mitochondria, the presence of an efficient proteolytic system might overcome the costly process of editing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by NIHES 005707 and 012691. We thank Professor Quinton Rogers for reading the manuscript and for providing us with excellent comments. We thank Mr. Andrew Almendares for his technical assistance.

REFERENCES

1. Attardi G. Animal mitochondrial DNA: an extreme example of genetic economy. *Int Rev Cytol.* 1985; 93:93–145. [PubMed: 3891661]
2. Baird GD. The Release of Amino Acids from Rat-Liver Mitochondrial Extract. *Biochim Biophys Acta.* 1964; 93:293–303. [PubMed: 14251307]
3. Ferdinand W, Bartley W, Broomhead V. Amino acid production in isolated rat liver mitochondria. *Biochem J.* 1973; 134:431–436. [PubMed: 16742802]
4. Truman DE, Korner A. Incorporation of amino acids into the protein of isolated mitochondria. A search for optimum conditions and a relationship to oxidative phosphorylation. *Biochem J.* 1962; 83:588–596. [PubMed: 13922697]
5. Giulivi C, Poderoso JJ, Boveris A. Production of nitric oxide by mitochondria. *J Biol Chem.* 1998; 273:11038–11043. [PubMed: 9556586]
6. Elfering SL, Haynes VL, Traaseth NJ, Ettl A, Giulivi C. Aspects, mechanism, and biological relevance of mitochondrial protein nitration sustained by mitochondrial nitric oxide synthase. *Am J Physiol Heart Circ Physiol.* 2004; 286:H22–H29. [PubMed: 14527943]
7. Sims NR. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. *J Neurochem.* 1990; 55:698–707. [PubMed: 2164576]
8. Chance B, Williams GR. A simple and rapid assay of oxidative phosphorylation. *Nature.* 1955; 175:1120–1121. [PubMed: 14394122]
9. Strydom DJ, Cohen SA. Comparison of amino acid analyses by phenylisothiocyanate and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate precolumn derivatization. *Anal Biochem.* 1994; 222:19–28. [PubMed: 7856847]
10. Cohen SA, De Antonis KM. Applications of amino acid derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Analysis of feed grains, intravenous solutions and glycoproteins. *J Chromatogr A.* 1994; 661:25–34. [PubMed: 8136907]
11. Ross-Inta CM, Z Y-F, Giulivi C. Threonine-deficiency diets induced changes in hepatic bioenergetics. *J Biol Chem.* 2007 submitted.
12. Semon BA, Leung PM, Rogers QR, Gietzen DW. Plasma and brain ammonia and amino acids in rats measured after feeding 75% casein or 28% egg white. *J Nutr.* 1989; 119:1583–1592. [PubMed: 2600663]
13. Semon BA, Leung PM, Rogers QR, Gietzen DW. Plasma ammonia, plasma, brain and liver amino acids and urea cycle enzyme activities in rats fed ammonium acetate. *J Nutr.* 1989; 119:166–174. [PubMed: 2918387]

14. Werkheiser WC, Bartley W. The study of steady-state concentrations of internal solutes of mitochondria by rapid centrifugal transfer to a fixation medium. *Biochem J.* 1957; 66:79–91. [PubMed: 13426112]
15. Birt LM, Bartley W. The pyridine nucleotide metabolism of mitochondria incubated with and without added substrates and metabolic inhibitors. *Biochem J.* 1960; 76:427–438. [PubMed: 16748831]
16. Chomyn A. In vivo labeling and analysis of human mitochondrial translation products. *Methods Enzymol.* 1996; 264:197–211. [PubMed: 8965693]
17. Smirnov VY, Razvodovskii YE, Doroshenko EM. Metabolic effects of mixture containing branched-chain amino acids and taurine during subchronic poisoning with barbiturates. *Bull Exp Biol Med.* 2004; 137:443–446. [PubMed: 15455113]
18. Fashakin JB, F P. Effect of dietary protein restriction on urinary nitrogen loss and tissue free amino acid patterns in mature rats. *Nutrition Research.* 1987; 7:285–298.
19. Shear L. Internal redistribution of tissue protein synthesis in uremia. *J Clin Invest.* 1969; 48:1252–1257. [PubMed: 5794248]
20. Braham JE, Bressani R. Effect of different levels of gossypol on transaminase activity, on nonessential to essential amino acid ratio, and on iron and nitrogen retention in rats. *J Nutr.* 1975; 105:348–355. [PubMed: 1117346]
21. Steffen M, Sarkela TM, Gybina AA, Steele TW, Trasseth NJ, Kuehl D, Giulivi C. Metabolism of S-nitrosoglutathione in intact mitochondria. *Biochem J.* 2001; 356:395–402. [PubMed: 11368766]
22. Smith CV, Jones DP, Guenther TM, Lash LH, Lauterburg BH. Compartmentation of glutathione: implications for the study of toxicity and disease. *Toxicol Appl Pharmacol.* 1996; 140:1–12. [PubMed: 8806864]
23. Wahllander A, Soboll S, Sies H, Linke I, Muller M. Hepatic mitochondrial and cytosolic glutathione content and the subcellular distribution of GSH-S-transferases. *FEBS Lett.* 1979; 97:138–140. [PubMed: 761610]
24. Hlavacova N, Bakos J, Jezova D. Differences in home cage behavior and endocrine parameters in rats of four strains. *Endocr Regul.* 2006; 40:113–118. [PubMed: 17201584]
25. Walberer M, Stolz E, Muller C, Friedrich C, Rottger C, Blaes F, Kaps M, Fisher M, Bachmann G, Gerriets T. Experimental stroke: ischaemic lesion volume and oedema formation differ among rat strains (a comparison between Wistar and Sprague-Dawley rats using MRI). *Lab Anim.* 2006; 40:1–8. [PubMed: 16460584]
26. Geis RB, Diel P, Degen GH, Vollmer G. Effects of genistein on the expression of hepatic genes in two rat strains (Sprague-Dawley and Wistar). *Toxicol Lett.* 2005; 157:21–29. [PubMed: 15795090]
27. Blemings KP, Crenshaw TD, Benevenga NJ. Mitochondrial lysine uptake limits hepatic lysine oxidation in rats fed diets containing 5 20 or 60% casein. *J Nutr.* 1998; 128:2427–2434. [PubMed: 9868191]
28. Alberti KG, Bartley W. The localization of proteolytic activity in rat liver mitochondria and its relation to mitochondrial swelling and aging. *Biochem J.* 1969; 111:763–776. [PubMed: 5783477]
29. Soboll S, Sholz R, Heldt HW. Subcellular Metabolite Concentrations. Dependence of Mitochondrial and Cytosolic ATP Systems on the Metabolic State of Perfused Rat Liver. *European Journal of Biochemistry.* 1978; 87:377–390. [PubMed: 668699]
30. Rogers Q. The nutritional and metabolic effects of amino acid imbalances. *Protein Metab. Nutr.* 1976; 16:279–301.
31. Bonnefond L, Fender A, Rudinger-Thirion J, Giege R, Florentz C, Sissler M. Toward the full set of human mitochondrial aminoacyl-tRNA synthetases: characterization of AspRS and TyrRS. *Biochemistry.* 2005; 44:4805–4816. [PubMed: 15779907]
32. King MP, Attardi G. Post-transcriptional regulation of the steady-state levels of mitochondrial tRNAs in HeLa cells. *J Biol Chem.* 1993; 268:10228–10237. [PubMed: 7683672]
33. Ulrich AK, Li LY, Parker J. Codon usage, transfer RNA availability and mistranslation in amino acid starved bacteria. *Biochim Biophys Acta.* 1991; 1089:362–366. [PubMed: 1713477]
34. Chomyn, AAG. Heterogeneous efficiencies of mRNA translation in human mitochondria; Cytochrome Syst.: *Mol. Biol. Bioenerg.*, [Proc. UNESCO Int. Symp.]; 1987. p. 145-152.

35. Hatefi, Y., Ragan, CI., Galante, YM. The enzymes and the enzyme complexes of the mitochondrial oxidative phosphorylation system. In: Martonosi, A., editor. *The Enzymes of Biological Membranes*. Vol. 1.4. New York: Plenum Press; 1985. p. 1-70.
36. Giulivi C. Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem J*. 1998; 332(Pt 3):673–679. [PubMed: 9620869]
37. Sonoda T, Tatibana M. Purification of N-acetyl-L-glutamate synthetase from rat liver mitochondria and substrate and activator specificity of the enzyme. *J. Biol. Chem*. 1983; 258:9839–9844. [PubMed: 6885773]
38. Gong J, Hunter GA, Ferreira GC. Aspartate-279 in Aminolevulinate Synthase Affects Enzyme Catalysis through Enhancing the Function of the Pyridoxal 5'-Phosphate Cofactor. *Biochemistry*. 1998; 37:3509–3517. [PubMed: 9521672]
39. Kunin AS, Tannen RL. Regulation of glutamate metabolism by renal cortical mitochondria. *Am J Physiol*. 1979; 237:F55–F62. [PubMed: 37743]
40. Schoolwerth AC, LaNoue KF. The role of microcompartmentation in the regulation of glutamate metabolism by rat kidney mitochondria. *J Biol Chem*. 1980; 255:3403–3411. [PubMed: 7364748]
41. Schaffer S, Solodushko V, Azuma J. Taurine-deficient cardiomyopathy: role of phospholipids, calcium and osmotic stress. *Adv Exp Med Biol*. 2000; 483:57–69. [PubMed: 11787642]
42. Lourenco R, Camilo ME. Taurine: a conditionally essential amino acid in humans? An overview in health and disease. *Nutr Hosp*. 2002; 17:262–270. [PubMed: 12514918]
43. Militante JD, Lombardini JB. Taurine: evidence of physiological function in the retina. *Nutr Neurosci*. 2002; 5:75–90. [PubMed: 12000086]
44. Pion PD, Kittleson MD, Rogers QR, Morris JG. Myocardial failure in cats associated with low plasma taurine: a reversible cardiomyopathy. *Science*. 1987; 237:764–768. [PubMed: 3616607]
45. Umeda N, Suzuki T, Yukawa M, Ohya Y, Shindo H, Watanabe K, Suzuki T. Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. *J Biol Chem*. 2005; 280:1613–1624. [PubMed: 15509579]
46. Suzuki T, Suzuki T, Wada T, Saigo K, Watanabe K. Taurine as a constituent of mitochondrial tRNAs: new insights into the functions of taurine and human mitochondrial diseases. *Embo J*. 2002; 21:6581–6589. [PubMed: 12456664]
47. Islam MM, Wallin R, Wynn RM, Conway M, Fujii H, Mobley JA, Chuang DT, Hutson SM. A Novel Branched-chain Amino Acid Metabolite: Protein-protein interactions in a supramolecular complex. *J. Biol. Chem*. 2007; 282:11893–11903. [PubMed: 17314104]
48. Blemings KP, Crenshaw TD, Swick RW, Benevenga NJ. Lysine-alpha-ketoglutarate reductase and saccharopine dehydrogenase are located only in the mitochondrial matrix in rat liver. *J Nutr*. 1994; 124:1215–1221. [PubMed: 8064371]
49. Mihalik SJ, Rhead WJ. Species variation in organellar location and activity of L-pipecolic acid oxidation in mammals. *J Comp Physiol [B]*. 1991; 160:671–676.
50. Fjellstedt TA, Robinson JC. Properties of partially purified saccharopine dehydrogenase from human placenta. *Arch Biochem Biophys*. 1975; 171:191–196. [PubMed: 242268]
51. Fjellstedt TA, Robinson JC. Purification and properties of L-lysine-alpha-ketoglutarate reductase from human placenta. *Arch Biochem Biophys*. 1975; 168:536–548. [PubMed: 1169916]
52. Hutzler J, Dancis J. Lysine-ketoglutarate reductase in human tissues. *Biochim Biophys Acta*. 1975; 377:42–51. [PubMed: 235294]
53. Hutzler J, Dancis J. Conversion of lysine to saccharopine by human tissues. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 1968; 158:62–69. [PubMed: 4385118]
54. Kemper EL, Neto GC, Papes F, Moraes KC, Leite A, Arruda P. The role of opaque2 in the control of lysine-degrading activities in developing maize endosperm. *Plant Cell*. 1999; 11:1981–1994. [PubMed: 10521527]
55. Noda C, Ichihara A. Control of Ketogenesis from Amino Acids. IV. Tissue Specificity in Oxidation of Leucine, Tyrosine, and Lysine. *J Biochem (Tokyo)*. 1976; 80:1159–1164. [PubMed: 1002682]
56. Papes F, Kemper EL, Cord-Neto G, Langone F, Arruda P. Lysine degradation through the saccharopine pathway in mammals: involvement of both bifunctional and monofunctional lysine-degrading enzymes in mouse. *Biochem J*. 1999; 344(Pt 2):555–563. [PubMed: 10567240]

57. Bate AJ, Dickson AJ. Relative importance of physiological precursors for ketogenesis in the nutritional homeostasis of the development of the embryonic chick. *Biochim Biophys Acta*. 1987; 924:7–12. [PubMed: 3828397]
58. Duszynski J, Mueller G, LaNoue K. Microcompartmentation of aspartate in rat liver mitochondria. *J Biol Chem*. 1978; 253:6149–6157. [PubMed: 681344]
59. Litt M, Kabat D. Studies of transfer ribonucleic acids and of hemoglobin synthesis in sheep reticulocytes. *J Biol Chem*. 1972; 247:6659–6664. [PubMed: 5076772]
60. Smith DW, McNamara AL. Specialization of rabbit reticulocyte transfer RNA content for hemoglobin synthesis. *Science*. 1971; 171:577–579. [PubMed: 5539722]
61. Dong H, Nilsson L, Kurland CG. Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. *J Mol Biol*. 1996; 260:649–663. [PubMed: 8709146]
62. Emilsson V, Kurland CG. Growth rate dependence of transfer RNA abundance in *Escherichia coli*. *Embo J*. 1990; 9:4359–4366. [PubMed: 2265611]
63. Emilsson V, Naslund AK, Kurland CG. Growth-rate-dependent accumulation of twelve tRNA species in *Escherichia coli*. *J Mol Biol*. 1993; 230:483–491. [PubMed: 7681880]
64. Yamao F, Andachi Y, Muto A, Ikemura T, Osawa S. Levels of tRNAs in bacterial cells as affected by amino acid usage in proteins. *Nucleic Acids Res*. 1991; 19:6119–6122. [PubMed: 1956771]
65. Ikemura T. Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. *J Mol Biol*. 1981; 146:1–21. [PubMed: 6167728]
66. Ikemura T. Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and *Escherichia coli* with reference to the abundance of isoaccepting transfer RNAs. *J Mol Biol*. 1982; 158:573–597. [PubMed: 6750137]
67. Ikemura T, Ozeki H. Codon usage and transfer RNA contents: organism-specific codon-choice patterns in reference to the isoacceptor contents. *Cold Spring Harb Symp Quant Biol*. 1983; 47 Pt 2:1087–1097. [PubMed: 6345068]
68. Atkins JF, Gesteland RF, Reid BR, Anderson CW. Normal tRNAs promote ribosomal frameshifting. *Cell*. 1979; 18:1119–1131. [PubMed: 391405]
69. Weiss WA, Friedberg EC. Normal yeast tRNA(CAGGln) can suppress amber codons and is encoded by an essential gene. *J Mol Biol*. 1986; 192:725–735. [PubMed: 3295253]
70. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature*. 1981; 290:457–465. [PubMed: 7219534]
71. Roy H, Ling J, Alfonzo J, Ibba M. Loss of editing activity during the evolution of mitochondrial phenylalanyl-tRNA synthetase. *J Biol Chem*. 2005; 280:38186–38192. [PubMed: 16162501]
72. Brindefalk B, Viklund J, Larsson D, Thollesson M, Andersson SG. Origin and evolution of the mitochondrial aminoacyl-tRNA synthetases. *Mol Biol Evol*. 2007; 24:743–756. [PubMed: 17182897]
73. Nakai T, Yasuhara T, Fujiki Y, Ohashi A. Multiple genes, including a member of the AAA family, are essential for degradation of unassembled subunit 2 of cytochrome c oxidase in yeast mitochondria. *Mol Cell Biol*. 1995; 15:4441–4452. [PubMed: 7623837]
74. Weber ER, Hanekamp T, Thorsness PE. Biochemical and functional analysis of the YME1 gene product, an ATP and zinc-dependent mitochondrial protease from *S. cerevisiae*. *Mol Biol Cell*. 1996; 7:307–317. [PubMed: 8688560]
75. Desautels M, Goldberg AL. Liver mitochondria contain an ATP-dependent, vanadate-sensitive pathway for the degradation of proteins. *Proc Natl Acad Sci U S A*. 1982; 79:1869–1873. [PubMed: 7043466]
76. Filho JC, Hazel SJ, Anderstam B, Bergstrom J, Lewitt M, Hall K. Effect of protein intake on plasma and erythrocyte free amino acids and serum IGF-I and IGFBP-1 levels in rats. *Am J Physiol*. 1999; 277:E693–E701. [PubMed: 10516129]
77. Bauerly KA, Storms DH, Harris CB, Hajizadeh S, Sun MY, Cheung CP, Satre MA, Fascetti AJ, Tchapanian E, Rucker RB. Pyrroloquinoline quinone nutritional status alters lysine metabolism

- and modulates mitochondrial DNA content in the mouse and rat. *Biochim Biophys Acta*. 2006; 1760:1741–1748. [PubMed: 17029795]
78. Baum JI, Layman DK, Freund GG, Rahn KA, Nakamura MT, Yudell BE. A reduced carbohydrate, increased protein diet stabilizes glycemic control and minimizes adipose tissue glucose disposal in rats. *J Nutr*. 2006; 136:1855–1861. [PubMed: 16772449]
79. Watanabe A, Higashi T, Sakata T, Nagashima H. Serum amino acid levels in patients with hepatocellular carcinoma. *Cancer*. 1984; 54:1875–1882. [PubMed: 6090002]
80. Hagman M, Eriksson T, Kitson KE. Similar effects of ethanol and tert-butanol on amino acid concentrations in rat serum and liver. *Alcohol Clin Exp Res*. 1993; 17:299–303. [PubMed: 8488972]
81. Soley M, Alemany M. A rapid method for the estimation of amino acid concentration in liver tissue. *J Biochem Biophys Meth*. 1980; 2:207–211. [PubMed: 7419868]
82. Cheung CW, Cohen NS, Rajman L. Channeling of urea cycle intermediates in situ in permeabilized hepatocytes. *J Biol Chem*. 1989; 264:4038–4044. [PubMed: 2917987]
83. Cohen NS, Cheung CW, Rajman L. Channeling of extramitochondrial ornithine to matrix ornithine transcarbamylase. *J Biol Chem*. 1987; 262:203–208. [PubMed: 3098738]
84. Srere PA. Enzyme concentrations in tissues. *Science*. 1967; 158:936–937. [PubMed: 6054167]
85. Srere PA, Sumegi B. Organization of the mitochondrial matrix. *Adv Exp Med Biol*. 1986; 194:13–25. [PubMed: 3529854]
86. Srivastava DK, Bernhard SA. Metabolite transfer via enzyme-enzyme complexes. *Science*. 1986; 234:1081–1086. [PubMed: 3775377]
87. Posakony JW, England JM, Attardi G. Mitochondrial growth and division during the cell cycle in HeLa cells. *J Cell Biol*. 1977; 74:468–491. [PubMed: 885911]
88. Schwerzmann K, Cruz-Orive LM, Eggman R, Sanger A, Weibel ER. Molecular architecture of the inner membrane of mitochondria from rat liver: a combined biochemical and stereological study. *J Cell Biol*. 1986; 102:97–103. [PubMed: 2867101]
89. Klingenberg, MPE. Structural and functional compartmentation in mitochondria; *Regul. Metab. Processes Mitochondria, Proc. Symp*; 1966. p. 180-201.
90. Institute for Laboratory Animal Research. Nutrient requirements of laboratory animals. 4th. Washington DC: National Academy Press; 1995. p. 11-59.

FIGURE 1. Correlation of free amino acid concentrations in mitochondria and amino acid abundance in mtDNA-encoded proteins

The total pool of mitochondrial amino acids (black bars) was assumed to be identical to that of liver cytosol (from Table II); free amino acids (from Table I; grey bars); amino acids used for mitochondrial protein synthesis were calculated as described in the text (white bars); other functions (red bars).

FIGURE 2. Dependence of free amino acids in mitochondria with amino acid content in mtDNA-encoded proteins

A, All amino acids; B: Excluding amino acids whose concentration would mainly sustain protein synthesis (inferred from Fig. 1).

TABLE I

Free amino acids in rat liver mitochondria *

Amino acid	(nmol/mg protein)
Ala	2.63
Arg	1.29
Asp	1.00
Asn	1.23
Cys	0.08
Glu	0.34
Gln	1.21
Gly	1.23
His	0.67
Ile	1.89
Leu	4.28
Lys	2.55
Met	1.38
Phe	2.07
Pro	0.21
Ser	1.81
Thr	1.33
Trp	0.29
Tyr	1.28
Val	1.48
Total	28.25
Others	
GSH	7.75
GSSG	0.73
Tau	0.15
Orn	0.99
Citrulline	0.00
Anserine	0.00
Carnosine	0.00
Sarcosine	0.00
1-Me-His	0.00
3-Me-His	0.00
Hydroxy-Pro	0.00
L- α -aminobutyric acid	0.00
Total	37.87

*The values represent the mean of 3 independent experiments with SD = 10%.

TABLE II

Pool of free amino acids and their derivatives in rat serum, liver, and liver mitochondria *

Amino acids & derivatives	Serum	Liver	Mitochondria **
		(mM)	
<i>Essential</i>			
His	0.09	0.57	0.80
Ile	0.10	0.81	2.27
Leu	0.15	1.57	5.16
Lys	0.78	1.87	3.08
Met	0.06	0.50	1.66
Phe	0.07	0.67	2.49
Thr	0.54	1.43	1.61
Trp	0.02	0.15	0.35
Val	0.21	1.46	1.79
<i>EAA Total</i>	<i>2.02</i>	<i>9.03</i>	<i>19.21</i>
<i>Nonessential</i>			
Ala	0.63	4.27	3.17
α -Amino-butyrate	0.01	0.00	0.04
Anserine	0.00	0.00	0.00
Arg [†]	0.19	0.01	1.55
Asn	0.23	0.46	1.48
Asp	0.02	2.02	1.20
Carnosine	0.00	0.00	0.00
Citrulline	0.10	0.02	0.00
Cys	0.005	0.04	0.07
Gln	0.73	0.57	1.46
Glu	0.12	3.13	0.41
Gly	0.38	2.41	1.48
HO-Pro	0.06	0.31	0.00
1-Me-His	0.01	0.01	0.00
3-Me-His	0.00	0.00	0.00
Orn	0.06	1.24	1.19
Pro	0.19	1.14	0.25
Sarcosine	0.02	0.003	0.00
Ser	0.40	1.95	2.18
Tau	0.21	2.38	0.18
Tyr	0.14	0.55	1.55
<i>NEAA Total</i>	<i>3.51</i>	<i>20.51</i>	<i>16.21</i>
EAA:NEAA ***	0.57	0.44	1.18
Gly/Ser	0.95	1.24	0.68

Amino acids & derivatives	Serum	Liver	Mitochondria **
Val/Gly	0.55	0.61	1.21
Tyr/Phe	2.0	0.80	0.60
AA	0.23	1.37	4.39
BCAA	0.46	3.84	9.22
AA/BCAA	0.50	0.36	0.48
K,G,T/BCAA	3.70 (2.52)	1.50 (1.11)	0.67 (0.49)

* Samples from serum, liver, and liver mitochondria were processed as described under Materials and Methods. Numbers in bold are those whose concentrations were higher or similar than those found in the mitochondrial matrix. The numbers are the average of 3 determinations with SD 10%. Serum values were within those published by others using either rat plasma [18, 76–78] or serum [79]; rat liver values were within those published by others [17, 18, 80] and comparable to those obtained with flash-frozen livers [81].

Abbreviations: AA, aromatic amino acids; BCAA, branched-chain amino acids; K,G,T, Lys, Gly, and Thr.

Numbers between parentheses indicate Lys plus Gly only.

** The amino acid concentrations indicated in this study were taken as *homogenously distributed* throughout the mitochondrial matrix. If kinetic or functional studies were to be performed with these concentrations, other biological settings should be considered that might undermine the concept of “concentration of free amino acid”. For instance, some of these situations are substrate channeling (as it is described for the urea cycle; [82, 83], microcompartmentation (as it was described for Asp and Glu; [40, 58], and diffusion of metabolites. In the last term, concepts of “free water”, “bound water”, and density of mitochondrial matrix become interconnected [84, 85]. Thus, mechanisms of metabolite transfer between enzymes will significant differ from that usually assumed where dissociation and random diffusion of metabolite through the aqueous environment is responsible for the transfer to the next enzyme site [86] Furthermore, this situation is exacerbated in mitochondria, where essentially it seems difficult to visualize any “free water” in the matrix when the relatively high protein [6], metabolite concentrations, and their hydration shell are taken into account. To calculate the concentration of amino acids in mitochondria, an average value for mitochondrial matrix volume was obtained ($0.83 \pm 0.04 \mu\text{L}/\text{mg}$ protein) from values reported by different laboratories using various techniques. The volume of the cytoplasmic compartment was based on the cytoplasmic compartment occupying 72% of a cell volume of 4.9 pL [87]. The volume of the mitochondrial compartment was calculated from the percentage of the cytoplasmic compartment occupied by mitochondria (10.5%; [87] and the percentage of mitochondria occupied by the matrix (64%; [88]). Using this approach, 4.8% of the tissue volume should be mitochondrial matrix volume (1 g liver = 48 μL mitochondrial matrix). Using our isolation procedure, from 1 g liver we obtain -in average- 30 mg mitochondrial protein (although if purified, is about 15 mg). Then the matrix volume to mitochondrial protein ratio would be 1.6 $\mu\text{L}/\text{mg}$. Werkheiser and Bartley [14] showed that only 60% of intramitochondrial water was permeable to sucrose. Thus, the corrected accessible water would be 0.96 $\mu\text{L}/\text{mg}$ mitochondrial protein. Others have reported a value of 0.8 $\mu\text{L}/\text{mg}$ protein [29, 89], 0.74 $\mu\text{L}/\text{mg}$ [58], 0.854 $\mu\text{L}/\text{mg}$ [40] using other approaches, in close agreement with the value found above.

*** To be able to compare EAA:NEAA ratios with values published before, Arg has not been included as an essential amino acid, even it is considered essential for growing rats [90]. If included, no significant differences would had been found in the EAA:NEAA ratios or in the final conclusions.