Tissue-specific changes in RNA synthesis in vivo during anoxia in crucian carp

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Smith, Richard W., Dominic F. Houlihan, Göran E. Nilsson, and Julie Alexandre. Tissue-specific changes in RNA synthesis in vivo during anoxia in crucian carp. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R690–R697, 1999.—The overall energy budget for protein synthesis (i.e., transcription plus translation) is thought to consist of fixed and variable components, with RNA synthesis accounting for the former and protein synthesis the latter. During anoxia, the downregulation of protein synthesis (i.e., the variable component), to reduce energetic demand, is an important aspect of survival in crucian carp. The present study examines RNA synthesis during anoxia by labeling with [3H]uridine. A novel synthesis rate calculation is presented, which allows for the tissue-specific salvage of uridine, with synthesis rates finally expressed relative to DNA. After 48 h anoxia, the decline (29%) in brain RNA synthesis and increases in the heart and liver (132 and 871%, respectively) support known RNA functions during hypoxic/anoxic survival. This study provides evidence that, in an anoxia-tolerant species, survival mechanisms involving RNA are able to operate because tissue-specific restructuring of the RNA synthesis process enables fixed synthesis costs to be maintained; this may be as vital to survival as exploiting the variable energetic demand of protein synthesis.

AN ABILITY TO USE anaerobic glycolysis to fully compensate for a stop in oxidative ATP production is one reason why the crucian carp, Carassius carassius, is one of the very few vertebrates able to withstand prolonged exposure to anoxia. Anaerobic glycolysis is the only important route for ATP production during anoxia and yields only a fraction of the ATP produced from oxidative respiration, yet protein synthesis can account for between 20 and 79% of the respiratory energy budget in fish (reviewed in Ref. 31). Recent results indicate that downregulation of protein synthesis in the liver and white muscle, while simultaneously retaining synthesis in the brain, enables the crucian carp to make a 40% reduction in protein synthesis in whole body terms, as well as ensuring neuronal survival (32). Because anoxic survival is ultimately limited by the depletion of liver glycogen (21), the ability to reduce the energetic demands associated with protein synthesis must be an important aspect of survival strategies used during prolonged anoxia (32).

The same study (32) also indicated that events upstream of translation may be involved in anoxic survival. Downregulation of protein synthesis in the heart, liver, and muscle was associated with reductions in RNA concentrations (µg RNA/mg protein) or RNA translational efficiency (mg protein synthesized·µg RNA−1·day−1) or both, whereas an increase in brain RNA translational efficiency enabled the maintenance of protein synthesis in this tissue despite a 50–60% reduction in micrograms RNA per milligram protein (32).

Anoxia-induced changes in overall RNA synthesis have received little experimental attention, and we do not know to what extent such pretranslational changes are responsible for the considerably altered protein synthesis rates displayed by anoxic crucian carp. Although neuronal survival is recognized as central to anoxia tolerance, this species depends not only on the maintenance of brain protein synthesis but also on the downregulation of muscle and liver protein metabolism (32). To complement our earlier study (32), we have now measured RNA synthesis in these tissues, in vivo, and thus attempted to answer questions on relative synthesis rates and the tissue-specific effects of anoxia on RNA synthesis and describe how any changes in RNA synthesis may contribute to the survival strategy of an anoxia-tolerant vertebrate.

MATERIALS AND METHODS

Fish, husbandry, and generation of anoxic conditions. Crucian carp, weighing 21.1 ± 0.9 g, were collected from a small pond 10 km north of Uppsala, Sweden. These fish were held in an internal 600-liter tank at the aquarium facility, Department of Limnology, University of Uppsala, for ~6 mo before experimentation. The holding tank was continuously supplied with local tap water (8–10°C) at a rate of 1 l/min. This facility incorporated a day-night cycle continuously and automatically adjusted to diurnal variations at latitude 51°N. Commercial fish pellets (EWOS ST 40, Astra-EWOS Sweden) at a 2% body wt ration were given once daily. The animals were starved for 24 h before introduction to experimental tanks and also throughout the experimental periods (21). In the experimental aquariums normoxic conditions were ensured by adequate aeration from a diaphragm-type air pump and air stone diffuser while anoxia was generated by bubbling nitrogen (type S, AGA Gas, Sweden) via a similar diffuser into the water (32). Dissolved oxygen was monitored using an OXI 96 oxygen electrode (WTW). Downregulation of protein synthesis occurs within 48 h of exposure to anoxia (32), and, to
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RESULTS

RNA concentrations. In normoxic tissues no differences in wet weight RNA concentrations were found in the brain, heart, and liver, whereas RNA expressed relative to DNA (mg/mg) was highest in the brain, with the heart and liver being similar (Fig. 1). After 48 h of anoxia, the concentration of RNA relative to wet weight declined in the brain and heart, but was unaffected in the liver. RNA/DNA also declined in the anoxic brain (Fig. 1A) but was found to increase in the heart (Fig. 1B) and remain unaffected in the liver (Fig. 1C).

Uridine uptake and recovery. Under normoxia, the total, i.e., that attributable to free as well as RNA-bound nucleotides, uridine recovered from the brain, heart, and liver accounted for ~79% of the injected dose (Table 1). This was not evenly distributed between the different tissues, with the majority being recovered from the liver. Although, after exposure to 48 h anoxia, the proportion of the labeling dose accumulated by the brain remained unchanged there was a reduction in uridine uptake by both the heart and liver (Table 1). Thus 59% of the injection dose could be accounted for from the anoxic tissues studied.

Uridine nucleotide specific activities. UMP was detected in all three tissues, whereas UDP was not detected in any sample and UTP was only measurable in the brain and liver (Table 1). In all tissues, uridine nucleotide specific activities stabilized within the 150-min time course (individual data not shown). There was a clear UMP specific activity ranking order under normoxia: liver > heart > brain (Table 1). Despite a decline in liver UMP specific activity, the same order was retained after exposure to anoxia. There was no difference between the normoxic UTP specific activity of the brain and liver. An increase in both after exposure to 48 h anoxia meant the anoxic UTP specific activities were also similar.

RNA activities. RNA labeling throughout the entire time course is described in Fig. 2. Between 20 and 40 min after injection, RNA activities were found to be linearly correlated with labeling time: r = 0.93, 0.93, 0.88 for normoxic brain (Fig. 2A), heart (Fig. 2B), and liver (Fig. 2C), and 0.90, 0.93, 0.62 for anoxic brain, heart and
liver, respectively. From 50 to 150 min labeling there were no significant changes with time in any tissue, suggesting a logarithmic incorporation during a protracted labeling period (Fig. 2). Exposure to 48 h anoxia had no effect on either the linear increase in RNA radioactivity with labeling time (dpm·µg RNA⁻¹·min⁻¹) or the mean steady-state RNA radioactivity levels attained (dpm/µg RNA) by any of the tissues studied. In both normoxia and anoxia these were greatest in the heart, followed by the brain, and lowest in the liver: 0.67 ± 0.03, 0.21 ± 0.03, 0.04 ± 0.01 dpm·µg RNA⁻¹·min⁻¹; 38.8 ± 2.3, 16.8 ± 0.8, and 3.6 ± 0.4 dpm/µg RNA for normoxic tissues; and 0.61 ± 0.04, 0.23 ± 0.04, and 0.03 ± 0.00 dpm·µg RNA⁻¹·min⁻¹; and 35.3 ± 2.9, 17.6 ± 1.1, and 2.5 ± 0.3 dpm/µg RNA for anoxic tissues, respectively.

RNA synthesis. RNA uridine incorporation relative to tissue RNA was equal in the brain (Fig. 3A) and heart (Fig. 3B); both were higher than in the liver (Fig. 3C). However, when expressed per milligram DNA, there was a clear ranking order between RNA uridine incorporation in the tissues studied: liver > heart > brain. Although in the brain (Fig. 3A) and liver (Fig. 3C) the effects of anoxia on uridine incorporation into RNA were similar irrespective of unit of measurement (i.e., a decrease in the brain but an increase in the liver), there was a disagreement in the heart (Fig. 3B); uridine incorporation into RNA per milligram RNA was unaffected, whereas incorporation into RNA per milligram DNA was found to increase.

Apart from the discrepancies listed above these units of measurement do not include a time factor and therefore, by definition, do not actually define a “rate” of RNA synthesis. However, from the RNA activity during the linear incorporation phase of the RNA labeling curve (Fig. 2) and on the assumption of a UMP molecular weight of 324.4 and UMP composition of RNA of 24.7% (30), we have been able to expand these original calculations and express RNA synthesis rates as micrograms RNA synthesized per microgram DNA per day (Fig. 4). These define tissue-specific normoxic RNA synthesis rates, i.e., brain > heart > liver and confirm that, in anoxic crucian carp, heart (Fig. 4B) and liver (Fig. 4C) RNA synthesis were increased, whereas, in the brain (Fig. 4A), RNA synthesis was reduced.

DISCUSSION

RNA synthesis and anoxic survival. Clearly neuronal survival is central to the survival of crucian carp. The results from the present study agree with in vitro data that demonstrate enhanced neuron survival after treat-

![Figure 1](http://ajpregu.physiology.org/)
inhibition of RNA synthesis can be explained as a protective mechanism.

In addition to the brain, the translational physiology of the liver has also been shown to be a major contributor to anoxia tolerance (32). A reduction in the supply of newly synthesized proteins is detrimental to rRNA survival (9) and, as is the case with heat shock, enhanced recovery depends on both the repair and replacement of preexisting ribosomes (4). Given the

Fig. 2. Labeling of total RNA in brain (A), heart (B), and liver (C) of crucian carp after injection with [3H]uridine under normoxia (○, dashed line) and after exposure to 48 h anoxia (●, solid line). Regression equations for complete labeling kinetics: normoxic brain y = -17.1 ± 4.4 + 16.9 ± 2.5 log x, r = 0.6; anoxic brain y = -21.6 ± 5.0 ± 4.7 ± 0.6 log x, r = 0.6; normoxic heart y = -22.6 ± 9.1 ± 34.0 ± 5.2 log x, r = 0.5; anoxic heart y = -26.6 ± 10.1 ± 31.6 ± 5.9 log x, r = 0.5; normoxic liver y = -5.6 ± 1.1 ± 47 ± 0.6 log x, r = 0.6; anoxic liver y = -3.3 ± 1.0 ± 31 ± 0.6 log x, r = 0.5; where y = RNA bound activity (dpm/µg RNA) and x = labeling time (min).

Fig. 3. Uridine incorporation into RNA in brain (A), heart (B), and liver (C) of crucian carp under normoxia (open bars) and after exposure to 48 h anoxia (solid bars). Data derived from 33 normoxic and 32 anoxic tissue samples. *Significant difference between anoxic data and appropriate normoxic control.
95% reduction in liver protein synthesis rates during anoxia (32), it was notable that the anoxic liver showed the highest relative increase in RNA synthesis. The majority of RNA is ribosomal, and the crucian carp must be capable of recovery with the resumption of normoxic conditions. The large increase in anoxic liver RNA synthesis rates could help to replace ribosomal RNA lost due to anoxia-induced degradation. This increase in liver RNA synthesis in fact coincides with elevated liver RNA/protein in the crucian carp during anoxia (32) and also with the increased production of translatable mRNA in the same organ of anoxia-tolerant turtles (15). It would therefore be of interest to know exactly which nucleotide sequences were being transcribed in the liver under anoxia.

In the heart an influx of water, as demonstrated in ischemic mammalian hearts (2), offers a likely explanation for the decline in wet weight RNA concentration. The increased RNA synthesis rate under anoxia must partially (at least) account for the increased RNA/DNA in this tissue but, because RNA/protein also has been shown to decline in the anoxic heart (32), this suggests an increased synthesis of RNA species other than rRNA. It is known that a number of specific cardiac proteins are synthesized in response to (mammalian) ischemia (reviewed in Ref. 11) and the release of specifically induced proteins is a response to ischemic preconditioning (36). One could propose that ischemic preconditioning is analogous to the seasonal exposure of the crucian carp to anoxia and presumably such proteins would require the rapid production of the appropriate RNA sequences. As is the case with the liver, it would be of interest to know exactly which RNA molecules were being transcribed and the contribution made by these to total heart RNA content. Additionally, because the uridine incorporation of RNA relative to RNA content and the labeling rate of total RNA are unchanged by anoxia, these data also indicate that RNA content is increasing in a manner proportional to the synthesis rate. This suggests that an increase in RNA stability (i.e., a reduction in RNA breakdown) in the heart contributes to anoxia tolerance and also to an increase in RNA relative to tissue wet weight.

These results demonstrate that, unlike protein synthesis (32), RNA synthesis is not downregulated in an anoxia-tolerant vertebrate. Nevertheless this study does provide evidence as to how the energetics of RNA synthesis could play a role in the physiological survival strategies outlined above. Recent evidence suggests the complete protein synthesis energy budget contains both a “variable” and “fixed” component (31). With increasing protein synthesis rates, the fixed component therefore accounts for a progressively smaller proportion of total energy expenditure. Hence increased synthesis rates are associated with reduced synthesis costs (31). RNA synthesis is thought to account for some, if not all, of the fixed cost component of protein synthesis (23). Therefore, by definition, the total cost of RNA synthesis must remain unchanged despite the anoxia-induced changes in protein synthesis observed in these tissues (32).

Unlike protein synthesis where, once the process starts, the substrate, i.e., amino acid, passes through a single intermediary phase, i.e., the tRNA-amino acid complex, to a single product, i.e., polypeptide, nucleotides have the potential to be directed to more than one product, possibly to RNA species other than those originally intended, or used as enzyme modulators or energy substrates. Protein synthesis downregulation acts on a single end point, whereas downregulation at any specific stage during RNA synthesis need not necessarily affect RNA production as a whole. RNA synthesis may represent an area where energy use is not so much downregulated as restructured.

Diverting RNA production costs in the liver toward an increase in a minority species, e.g., mRNA, which would have little effect on total RNA content (33), could ensure that overall a fixed amount of energy is expended on RNA synthesis as a whole.

Nucleotide precursor supply constitutes the greatest energetic demand within RNA metabolism (5). The current study provides some evidence concerning the energetic restructuring of this aspect of RNA synthesis. Although both involve a significant amount of energy, the salvage of exogenous nucleosides avoids the higher metabolic costs of intracellular de novo synthesis (28).
Higher rates of protein synthesis are associated with lower protein synthesis costs (31), and, under normal conditions, the protein synthesis ranking order, liver > heart > brain (32), corresponds to the extent of nucleotide salvage shown in the present study; tissues with higher protein synthesis rates place greater reliance on the least costly route of nucleotide supply.

At first consideration, it would appear reasonable to expect an increase in the salvage of exogenous nucleosides in all tissues after anoxic exposure, yet these data suggest there was no shift toward nucleoside salvage in the anoxic brain and heart and a reduction in salvage by the anoxic liver. In the liver, an increased synthesis of certain mRNAs would presumably not significantly affect the demand on nucleotide supply and, with an increased production of RNA species with a greater turnover rate, could also, via nucleotide recycling, reduce the necessity for exogenous salvage.

In the case of the brain, a reduction in RNA synthesis rate, as a direct survival strategy (discussed), incurs an energetic commitment to maintain de novo (i.e., more expensive) nucleotide synthesis to maintain a fixed energetic cost. As there is normally a balance between de novo and salvage pathways, which produces a nucleotide pool appropriate to requirements (28), there must be some explanation to account for the continuing dependence on de novo synthesis under anoxia. One could speculate that nucleotide salvage is relatively unreliable, e.g., diet dependent, whereas an energetically based preference for de novo production ensures the brain has a more constant supply.

Methodology. There is a difference of opinion regarding the kinetics of uridine movement through cell membranes. Some studies suggest the uptake rate is concentration dependent (13), whereas others provide evidence to the contrary (14). However, exogenous nucleoside salvage is a two-step process. Movement of an unmodified substrate (26) across the membrane is immediately followed by phosphorylation, thus “trapping” (35) the resultant nucleotide, with the latter being the rate-limiting phase (35). Thus internal pools do equilibrate with the exogenous uridine (34). Furthermore, any resulting increase in precursor concentration, arising from the uridine labeling dose, has only a limited effect on incorporation into RNA (17). Labeling concentrations of 100 μM uridine lead to equilibration within 250–300 s (13) and, with an extracellular uridine concentration of 1.0 mM, any possible changes in the precursor pool are minimized (24), thus ensuring a constant precursor supply. On the assumption that crucian carp possess a blood volume within the range thus far recorded for teleosts, i.e., 30–70 ml/kg body wt (6), in the present study this would result in blood uridine concentrations of between ~14 and 33 mM.

The mean HPLC retention times (± SE) for UMP and UTP were 3.359 ± 0.018 and 27.708 ± 0.040 min, respectively (min/max = 3.308/3.392 and 27.617/27.808 min, respectively). No radiation was detected in any fraction eluted out with these times. Specifically, none was detected before UMP elution, corresponding to nonphosphorylated uridine. Because intracellular phosphorylation is required to yield a uridine metabolite to which the cell membrane is impermeable (35), thus preventing reverse diffusion out of the cell (5), we can only conclude that, in the present study, any nonphosphorylated uridine was largely lost back to the extracellular space and therefore not present in detectable quantities in the intracellular fractions. This would mean that all of the detected radioactivity belonged to free or RNA-bound uridine nucleotides or their analogs, as opposed to nonmetabolized uridine. Although this in fact accounted for the majority of the injected labeling dose, the tissues studied comprise only ~11% of the total body weight of crucian carp. However, on a mass-specific basis, the brain, heart, and liver are ~36, 19, and 11 times, respectively, more metabolically active than white muscle (22), which constitutes the bulk of body tissue.

UTP was not detected in the heart, presumably because the UTP pools were extremely small and/or rapidly utilized for RNA synthesis. Additionally, nucleotide triphosphates are particularly unstable under hypoxic conditions (7). Therefore the acceptable alternative of total uridine nucleotide specific radioactivity, as an indicator of RNA precursor labeling (7), has been used in the calculation of RNA synthesis in this study.

Accurate nucleic acid measurement is pivotal to this study, and the potential problems have been summarized elsewhere (29). The nature of the biological material is known to present specific considerations (19), and, in the case of tissues from mice and rats, this has necessitated the development of a new analytic approach (29). However, the methodological considerations relevant to the present study have been addressed by established techniques. The combination of PCA at 4°C and 0.2 M, and the Potter-Elvehjem homogenizer, inhibits nuclease action and is optimal for removing acid-soluble compounds without the risk of solubilizing RNA (19). The following alkali extraction and subsequent 20% PCA treatment, therefore, effectively separates RNA from DNA and protein (19), resulting in a 95.4 (18) to 100% (19) recovery of RNA. RNA concentration based on UV absorption overcomes any colorimetric (carbohydrate reaction with orcinol) interference from glycogen (19), a particular consideration of the present study (refer to Ref. 32). This method of determining concentration is also possible because PCA does not interfere with UV light (19). In addition to glycogen the other main contaminant of RNA is likely to be protein breakdown products. However, spectral examination has shown an insignificant (i.e., <2%) peptide contamination of RNA extracted by this method (20) and the inclusion of dual UV absorbance (1) in fact eliminates interference from any hydrolyzed protein (1). This RNA extraction/concentration determination technique has recently been successfully compared with alternative assay methods; i.e., concentrations derived from reaction with orcinol and also based on fluorescence (18), and the use of the same method also means these RNA synthesis data can be directly compared with RNA content, relative to pro-
protein, and RNA translational efficiency data previously recorded in crucian carp (32).

Logarithmic increases in RNA labeling, with time, have been demonstrated in mammalian research, and the 50 min required to achieve maximum uridine labeling is similar to the 60 min evident in mice (16) and ground squirrels (5). The statistically insignificant changes in uridine nucleotide and RNA activity 50 min after injection suggest a steady state of nucleotide interchange between synthesized RNA and the nucleotide pool. Unlike the brain and heart, there was a small time interval between \(^{3}H\)uridine injection and the appearance of radiation in RNA. This can be explained by the slower rate of RNA synthesis within this tissue.

Expression of RNA synthesis rates relative to DNA quantifies RNA synthesis on a “per cell” basis (7), and it was of interest to note that, in the current study, DNA-based uridine incorporation (nmol incorporated into RNA/mg DNA; Fig. 3) compared favorably with RNA synthesis rates (\(\mu\)g RNA · \(\mu\)g DNA \(^{-1}\) day \(^{-1}\); Fig. 4).

The labeling kinetics of RNA present a complex situation, but this study clearly demonstrates the advantage that calculations of RNA synthesis based on RNA labeling relative to nucleotide precursor pool radioactivity have over the usage of RNA radioactivity only. The calculation of RNA synthesis rates from a stabilized nucleotide precursor pool and linear incorporation of radioactivity into RNA, during the initial 40 min of the RNA labeling time course, complies with similar criteria used in the investigation of protein synthesis (refer to Ref. 32). In this study, levels of radioactivity in RNA (dpm/\(\mu\)g RNA) were a poor indication of both normal tissue-specific RNA synthesis rates and of the effects of anoxia. These data demonstrate that, in addition to synthesis rate, RNA-bound activity is also influenced by the extent of exogenous nucleoside salvage.

In addition to the similarity in RNA labeling kinetics between this and previous studies, the liver uridine incorporation data, per milligram RNA (Fig. 3C), were also similar to the rat (7). Furthermore, from these data, it is possible to express brain RNA synthesis in terms of the percentage distribution of radioactivity between free UTP and RNA (5). With the use of this approach, the normoxic value, 7.1 ± 0.7% radioactivity in RNA, and the anoxia-induced decline, 2.0 ± 0.9% radioactivity in RNA, were similar to that in the hypoxia-tolerant ground squirrel (5). In contrast, the relatively low rates of liver RNA synthesis and the relatively high RNA/DNA in the brain and heart contradict similar data from the same tissues in rats (29). However, RNA/DNA has been defined as a per genome accumulation of RNA. As previously demonstrated (29) and despite these contradictions, in the present study the tissue ranking order for RNA synthesis rates mirrors that for RNA/DNA. Therefore, despite this partial contradiction, these data support the theory that larger cells, i.e., those with a higher RNA/DNA, synthesize more RNA than smaller cells (29).

Perspectives

The current study, together with the preceding investigation (32), demonstrates how the survival strategy of an anoxia-tolerant species operates within the energetic model that describes the overall process of protein metabolism. With the establishment of the role of translational (32) and transcriptional (this study) metabolism, a logical progression could be the investigation of pretranscriptional events and anoxic survival. For example, the data presented here are not mutually exclusive with the demonstration that nucleoside uptake is unaffected by hypoxia (16). Reductions in UMP specific activity in the anoxic liver could be accounted for by reduced intracellular phosphorylation. Such an explanation not only complies with this being the rate-limiting step in nucleoside salvage, but also with the evidence that shows that the majority of uridine uptake occurs via an energy-independent carrier (25). However, a second active system is also involved in uridine transport (10), albeit of a lesser quantity, and recent investigations have in fact shown that transport across the cell membrane is increased by ATP and its analogs (13). This suggests the opposite to be true when ATP supply is decreased. These investigations appear to show that both uptake systems work in tandem. Clearly a more specific study would be required to characterize tissue-specific uridine salvage under anoxia and if there is any pretranscriptional downregulation in an anoxia-tolerant species.

In conclusion, the present results demonstrate that, unlike protein synthesis, the crucian carp is unable to downregulate RNA synthesis when exposed to anoxia. Thus, when faced with a reduction in ATP supply, the present study confirms that metabolic downregulation occurs solely within the translational aspect of protein metabolism. Although (in whole body terms) this means there is no energetic saving associated with anoxic RNA synthesis, the tissue-specific changes in RNA synthesis rates are integral with other aspects of RNA metabolism during cellular survival. Overall protein synthesis costs comprise fixed and variable components, with RNA accounting for the former and protein synthesis the latter. Therefore the current study could demonstrate that tissue-specific survival mechanisms, which involve RNA, are possible because this fixed cost is maintained, despite any changes in synthesis rate; this being achieved by a redistribution of energy requirements within the RNA synthesis process. Thus, in addition the successful exploitation of the variable energetic component, i.e., downregulating protein synthesis (32), survival by an anoxia-tolerant species also depends on maintaining the fixed energetic cost attributable to RNA synthesis.

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