Protein Arginylation in a Partially Purified Fraction of 150k xg Supernatants of Axoplasm and Vertebrate Nerves

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Summary

Transfer RNA mediated posttranslational protein modification by arginine has been demonstrated *in vitro* in axoplasm extruded from the giant axons of squid, and following injuring to vertebrate nerves. In nerve and axoplasm the highest activity is found in a fraction of a 150k xg supernatant containing high molecular weight protein/RNA complexes but lacking molecules of < 5k Da. Arginylation (and protein modification by other amino acids) is not found in more purified, reconstituted fractions. The data are interpreted as indicating that it is critical to recover the reaction components in high molecular weight protein/RNA complexes in order to maintain maximum physiological activity. The level of arginylation is greatest in injured and regenerating vertebrate nerves compared with intact nerves, suggesting a role for these reactions in nerve injury/repair and during axonal growth.

Key words: Arginylation, posttranslational protein modification, axoplasm, injured nerves, regeneration, protein/RNA complexes

1. Introduction

Axoplasm extruded from the giant axon of squid contains relatively large quantities of a 76 nt (4S) RNA, some of which has been identified as transfer RNA (1-3). In vertebrate nerves, the biochemical components of axons are more difficult to identify because of their close association

with surrounding glia/Schwann cells. Nevertheless, a series of experiments have demonstrated large amounts of axonally transported 76nt RNA in regenerating goldfish optic and rat sciatic nerves and during growth of developing rat optic nerves (reviewed in Ref. 4). In growing vertebrate axons some axonal 76nt RNA is likely to be tRNA (5). A portion of the axonal tRNA in squid and elongating vertebrate axons functions in protein synthesis shown to take place in squid axoplasm (6) and in growing vertebrate axons (7).

In an attempt to ascribe function to the bulk of the 76nt RNA in growing axons, we tested the hypothesis that these RNAs were tRNAs and that they served not in protein synthesis, but as amino acid donors in the posttranslational modification of proteins. In rat brain extracts, only $tRNA^{Arg}$ has been shown to be able to posttranslationally modify proteins (8). While many of our experiments focused on $tRNA^{Arg}$, we have found that several other amino acids also could be incorporated posttranslationally into endogenous nerve proteins (3, 9). These reactions can be demonstrated *in vitro* only in partially purified, high molecular weight fractions of high speed supernatants from which molecules of <5.0 k Da had been removed (3,9). This activity is dependent on the physiological state of the tissue; i.e., nerve injury and nerve elongation *in vivo* result in large increases in these reactions when measured *in vitro* (9). Based on these data, we suggest critical roles for arginylation and other amino acid modifications of endogenous proteins in nerve injury/ repair and during growth of vertebrate axons.

2. Materials – The experiments below were performed in the late 1970s through the mid 1990s. The materials described are those available at the time.

2.1 Tissues

2.1. Axoplasm – Pure axoplasm, void of contamination from surrounding tissue, can be isolated from the giant axons of the stellate nerves of squid. Squid, (*Loligo pealii*) were obtained at the Marine Biological Laboratories in Woods Hole, MA.

About 5ul per axon of aAxoplasm is pooled and stored in 1.2 ml Eppendorf tubes at - 20^0C in 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 10 mM KCl, brought to 50% glycerol (to prevent samples from freezing). Based on squid availability from the daily catch, it is reasonable to collect axoplasm from 6-10 axons in a day.

Sciatic and optic nerves of rats – Male, Sprague-Dawley rats (~150 gms) were used in all experiments. Rats are anesthetized with ketamine / xylazine (100 mg/kg body weight) and sacrificed by an overdose of anesthesia and decapitation. Isolated nerve segments are placed in chilled buffer (100 mM Tris-HCl, pH 7.4, containing 250mM sucrose, and 6.0 mM dithiothreitol.)

2.2 Sample preparation

Squid axoplasm

1. On the day of the experiment, remove 100 ul of axoplasm (approx. 1.0 ug protein/ul) from -20^oC storage and transfer to a 1.2-ml conical Eppendorf tube. Keep samples at 4^0C during the entire procedure.

Rat sciatic and optic nerves

1. In anaesthetized rats, remove a one cm portion of nerve from: a) the site of injury (including the nerve segment proximal to the cell body and b) a segment distal to the injury. Place nerve segments on chilled glass slides.

2. Dissect the nerve segments as described in Methods.

3. Place the desheathed, minced nerve segment in 0.35 ml of ice cold buffer (100 mM Tris-HCl, pH 7.4, containing 250mM sucrose and 6.0 mM dithiothreitol) in a 1.2-ml conical Eppendorf tube.

2.3 Reactions

Reaction components contain approx. 0.5 - 1.0 ug protein/ul of partially purified axoplasm or rat nerve extracts, an equal volume of buffer (5 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, in 30 mM Tris-HCl (pH 7.4) and 5 ul (approx. 5 uCi) of ^{3}H-Arg or other ^{3}H labeled amino acids.

2.4 Determination of Protein Arginylation

The assay for the incorporation of ^{3}H-Arg into protein is done by precipitation in trichloro acetic acid (TCA) as described below.

3. Methods

3.1 Preparation of Tissue Samples

Squid Axoplasm

1. Live squid are used immediately after capture or are kept in tanks with running sea water for no longer than 2-3 days.

2. Squid are decapitated with a scissors and the stellate nerves containing the paired giant axons are identified with the aid of a dissecting microscope.

3. The axons, still connected to their cell bodies in the stellate ganglion, are removed intact.

4. The distal end of the axon is cut using dissecting scissors and axoplasm is extruded from the sheath by pressure, much like squeezing toothpaste from a tube. (for more detailed descriptions of this technique see Ref. 10, **Note 1**).

Rat sciatic and optic nerves-

Sciatic nerves:

1. Expose the superficial muscles of the upper leg with a longitudinal incision in the mid-thigh region.

2. Separate the deep muscles with blunt dissection to avoid bleeding.

3. Expose the sciatic nerve for about 4 cm distal from the sciatic notch.

4. Crush the sciatic nerve using curved jeweler's forceps (**Note 2**).

5. A translucent band in the otherwise opaque white nerve indicates that sciatic nerve axons have been cut.

Optic nerves:

1. Make a superficial incision in the sclera surrounding the back of the eye.

2. Rotate the eye forward.

3. Visualize the optic nerve using a dissecting microscope.

3, Identify the retinal artery (**Note 3**).

3. Crush the optic nerve (as described for sciatic nerves), making sure to avoid the retinal artery.

4. A translucent band in the otherwise opaque white nerve indicates that optic axons have been severed.

3.2 Preparation of the active fractions

Prepare the 150k xg supernatant as follows:

Axoplasm:

1. Remove 100 ul of axoplasm from -20^{0}C storage and transfer to a 1.2 ml Eppendorf tube.

2. Homogenize samples using a pestle made of dental cement (**Notes 4 and 5**).

2. Wash the Eppendorf tube with 60 ul of 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol.

3. Pool the wash with the homogenate.

4. Transfer the homogenate to a 200 ul capacity high-speed polypropylene centrifuge tube.

5. Centrifuge at 150k xg for 60 minutes at 4^{0}C in a Beckman Airfuge centrifuge.

Rat nerve segments:

1. Anaesthetize rats and remove nerve segments.

2. Place the nerve segment on a chilled glass slide.

3. Desheath the nerve using fine forceps and mince with a razor blade.

4. Homogenize 2 – 4 pooled nerve segments in 0.35 ml of 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol, in a 10 ml all-glass Ten Broeck homogenizer (**Note 5**).

5. Wash the homogenizing tube with 150 ul of 0.35 ml of 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose and 6 mM 2-mercaptoethanol.

6. Transfer the homogenate to a high-speed polypropylene centrifuge tube.

7. Centrifuge at 150k xg for 60 minutes at 4^{0}C.

Fractionation by Sephacryl S-200 chromatography

The 150k xg supernatant is fractionated by column chromatography on a Sephacryl S-200 column (2.5 mm X 300 mm; Pharmacia). The goal of this procedure is to collect a fraction containing high molecular weight protein/nucleic acids while removing molecules of < 5kDa (**Note 6)**.

For both axoplasm and vertebrate nerves:

1. Apply the 150k xg supernatant of tissue homogenates onto a Sephacryl S-200 column (4^0C). (other separation techniques can be used as long as the procedure removes molecules of < 5k Da. **(Note 7).**

2. Collect the effluent of the column to be used as the source of the reaction components.

3.3 (assay for posttranslational incorporation of amino acids into protein)

The void volume fraction of the S-200 column is used as the source of tRNA, aminoacylating enzymes, putative amino acid transferases, and endogenous acceptor proteins as components of HMW complexes. Keep this fraction on ice until incubation.

Axoplasm:

1. Add 15 ug protein (approx. 15ul) of the gel filtration column effluent to an incubation tube.

2. Add an equal volume of a reaction mixture (5 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, in 30 mM Tris-HCl (pH 7.4).

3. Add 3 - 5 ul (approx. 5 uCi) of ^{3}H-Arg or other tritium labeled amino acids.

4. Start reactions by transferring samples to a 37^{o}C water bath.

5. Incubate samples for 20 minutes at 37^0C.

6. Stop reactions by adding a volume of ice cold 10% TCA equal to the reaction volume, and mix well. (**Note 8**).

7. Place all samples in an ice bucket

3.4 Determination of Protein Arginylation (Note 9)

1. Leave reaction samples in ice for 30 minutes to allow RNA and proteins to form aggregates.

2. Centrifuge at 1,100 xg for 10 min. and remove supernatant.

3. Repeat until radioactivity in the cold TCA soluble fraction (free ^{3}H-amino acids) reaches background (4-5 washes).

4. Add 0.5 ml of 10% TCA to the cold TCA precipitate and transfer tubes to a boiling water bath for 20 minutes (this procedure hydrolyzes the aminoacyl-tRNA bond releasing amino acids bound to tRNA.)

5. Return samples to ice for 20 minutes.

6. Repeat steps 2 and 3 until the radioactivity in the cold TCA fraction reaches baseline.

7. Dissolve the precipitate in a tissue solubilizer and count in a liquid scintillation counter. This fraction contains the radioactive amino acid that had been covalently bound to protein **(Note 10).**

Closing comments: When these experiments were performed in the latter part of the 20th century, the only roles for an RNA of ~ 76nt (the RNA shown to be in abundance in squid axoplasm and axons and growth cones of regenerating - but not intact - vertebrate nerves; reviewed in Ref 4), was as tRNA, as an amino acid donor in protein synthesis or, as an Arg donor in posttranslational protein modification.

In recent years a new role for 76nt RNA s has been uncovered in the emergent field of RNA interference (reviewed in 11). Micro RNAs, 22nt RNAs that act to regulate translation of mRNAs (11), have been shown to be derived from 76nt precursors (pre-miRNAs) (12). The enzyme, Dicer, cleaves the 76nt precursor into the active 22 nt miRNA (11, 12). Recently, micro

RNAs have been reported to be present in axonal growth cones (13) and blocking specific miRNAs or disruption of the activity of Dicer can effect axonal guidance and the development of axonal polarity (13,14).

Based on these recent findings, we speculate that the abundant 76 nt RNA found in growing vertebrate axons is composed of: 1) tRNA that functions in classical axonal protein synthesis, 2) tRNAs involved in posttranslational protein modification as described in this volume, and 3) pre miRNAs that serve as precursors for miRNAs that regulate axon guidance.

4. Notes

Note 1. The dissection of the stellate nerves from the squid and the subsequent isolation of the giant axon and collection of axoplasm are described in detail elsewhere (Ref. 10). Also, YouTube videos demonstrating the procedure are available - http://www.google.com/search?client=safari&rls=en&q=squid+axon+dissection&ie=UTF-8&oe=UTF-8 . The procedure is probably best learned from a practitioner currently working with preparations of the giant axon.

In the preparation of axoplasm it is critical to keep extruded axoplasm separate from adaxonal glial contamination. To accomplish this, the isolated axon is placed on a slide covered with parafilm. The end of the axon is cut. As the axoplasm is pushed out, the axon sheath is pulled back. This leaves a 'bead' of about 5.0 ul of axoplasm.

Note 2. – Nerve crushes are performed by compressing the nerve in curved jeweller's forceps to a count of 10. Nerves are released and crushed again at the same site for an additional 10 seconds. Following the second crush the nerve is intact (proximal and distal segment are still

connected) but should turn translucent (from the usual opaque white) indicating that sciatic nerve axons have been transected.

Note 3. – It is critical to avoid the retinal artery when crushing the rat optic nerve, since cutting it will lead to the death of retinal ganglion cells and a physiological condition where no response to injury or regenerative growth is possible. If there is bleeding, the preparation should be discarded

Note 4. Volumes of axoplasm used per experiment are typically 100ul. Because of the small volume, homogenizing vessels can be constructed using conical 1.2 ml Eppendorf tubes and pestles made of dental cement. Pestles are made by placing dental cement in the bottom of an Eppendorf tube to the point where it just fills the tapered portion of the tube. A metal rod is placed in the center and the cement is allowed to harden. Once the cement forms, the Eppendorf tube can be broken away. The pestles are inert and can be washed and reused in subsequent homogenizations.

Note 5. Homogenization for axoplasm and sciatic nerves is performed by smooth up and down strokes of the pestle into the homogenate for 3 minutes while the sample is in an ice bucket. The degree of homogenization is critical and should be consistent among samples as over-homogenization leads to disruption of the high molecular weight protein-RNA complexes and a loss of physiological activity (see Refs. 9).

Note 6. In experiments in both axoplasm and vertebrate nerves we were not able to demonstrate posttranslational arginylation of protein in unfractionated 150k xg supernatants. However, reactions did take place in fractions containing high molecular weight nucleic acid/protein complexes but lacking molecules between 1k Da and 5 k Da (see Refs 3, 9).

Note 7. Gel filtration chromatography of the 150k xg supernatant is performed in order to remove small molecules from the fraction being assayed for posttranslational aminoacylation.

These small molecules inhibit the posttranslational addition of amino acids to protein. The mechanism of this inhibition has been investigated (15) but is not well understood.

Note 8. Controls for non-specific binding can be done in 3 separate ways: 1) Place the active fraction in a boiling water bath for 20 minutes (inactivating enzymes in the fraction) prior to incubation at 37^0C. These boiled control samples are incubated along with the experimental samples. 2) Add cold TCA to the samples before incubation, 3) run parallel samples using ^{3}H taurine as the labeled amino acid. Taurine, is a sulfonic amino acid not incorporated into any protein. In our experiments, the levels of taurine associated with protein following incubation and acid extraction were always negligible (see Refs. 3,9).

Note 9. Two important keys to demonstrating these reaction in tissue extracts should be noted: 1) the 'active fraction' must contain both RNA and protein. We monitored this by measuring absorbance at 260nm and 280 nm for each fraction as it came off the filtration column (see Ref. 9). 2) the 'active fraction' must be void of molecules of less than 5k Da. The best method to remove small molecules from the active fraction should be determined empirically for the tissue being studied.

Note 10. It is important to be sure that following incubation, the ^{3}H- label (being measured as amino acid incorporated into protein) is still part of the parent amino acid. Under the incubation and extraction conditions we used, we have demonstrated that ^{3}H remains associated with amino acids throughout these incubations (see Refs. 3,9).

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