The Use of Avidin as a Probe for the Distribution of Mitochondrial Carboxylases in Developing Chick Retina

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SUMMARY During development, the inner chick retina progresses from an aerobic to an anaerobic metabolic basis because of the lack of a vascular system. To investigate this process further, we have examined the expression and distribution of mitochondrial carboxylases. Because these enzymes use covalently bound biotin as a co-enzyme, we were able to develop a new detection protocol for mitochondria using avidin as a probe for the biotin. Chemiluminescent detection of bound avidin-peroxidase was used to examine a developmental series of extracts of retinas that had been separated by electrophoresis and blotted to nitrocellulose. Avidin-peroxidase, visualized with the sensitive peroxidase substrate True Blue, permitted detection in epoxy-embedded tissue sections. In the extracts, specific bands of approximate molecular weights 130 and 70 kD were found, corresponding to biotinylated subunits of several mitochondrial carboxylases. During development, the intensity of the bands decreases, although at different rates. In tissue sections, 8-day embryonic retinas display reaction product throughout the tissue, with higher local concentrations in the vitread and sclerad regions. During further development, the reaction product becomes segregated into bands at the borders of the plexiform layers. As the photoreceptors mature, stain becomes concentrated in the developing ellipsoids and the sclerad ends of Müller cells. (J Histochem Cytochem 46:177-183, 1998)

T he distinctive metabolic properties of the avian retina have been studied previously in this and other labs. Studies of the retinas of many vertebrate species have shown enzymes associated with glycolytic metabolism to be located in the inner retina, whereas oxidative enzymes localize to the outer retina (Lowry et al. 1956; Kuwabara and Cogan 1959a,b; Eranko et al. 1961). Anomeric studies in vitro have shown the avian retina in particular to have a lower respiratory rate than that of other species and an extremely high glycolytic rate (Krebs 1972). In a supportive finding, Hughes and colleagues (1972) found by means of electron microscopy that the respiratory organelle, the mitochondrion, is absent from all but a small portion of the adult pigeon retina. In our lab, a similar distribution of mitochondria has been shown in the adult chicken retina (Buono and Sheffield 1991a).

In this study we examined the properties during development of several mitochondrial enzymes in which biotin is a co-factor. Pyruvate carboxylase, methylcrotynyl CoA carboxylase, and propionyl CoA carboxylase are multi-subunit enzymes involved in the production of oxaloacetate, the degradation of leucine, and the breakdown of methionine, leucine, and fatty acids, respectively (Wood and Barden 1977; Lau et al. 1979; Bramwell 1987). The biotinylated subunits (approximate molecular masses of 130, 75, and 72 kD, respectively) of all three enzymes have been localized to the mitochondria of several mammalian tissues and cell lines, by several methods of centrifugation (Moss and Lane 1971; Mackall and Lane 1977; Petrelli et al. 1978; Chandler and Ballard 1986) and by immunohistochemical analysis (Gratzner et al. 1980). Because biotin is bound extraordinarily tightly by avidin, we have explored the use of labeled avidin as a mitochondrial tracer.

By using avidin-peroxidase and appropriate substrates, we found that in crude retinal cell extracts, biotin-containing proteins of the approximate molecular
weights of the biotinylated subunits of the carboxylases described above were present. Their quantity, as a percentage of total protein, was found to decline significantly with increasing age (Kuwabara and Cogan 1959a,b). In sections of retina of increasing embryonic age, the distribution of avidin labeling changes from generalized throughout the tissue in embryonic Day 8 to discrete bands in the outer retina at hatching.

Materials and Methods

All animal studies were performed in accordance with the ARVO recommendations for the use of animals in ophthalmic and vision research.

For Western blots, embryonic chicks (E8–E18) were sacrificed by decapitation and their retinas immediately dissected out in Tyrode’s (TCT; 0.15 M NaCl, 4 mM KCl, 0.01 M glucose, 0.4 mM NaH$_2$PO$_4$, 0.2 mM KH$_2$PO$_4$, 1 mM NaHCO$_3$, and 0.01 M ethylenediamine tetraacetic acid (EDTA), pH 7.4) on ice. Post-hatch (PH) chicks were sacrificed by decapitation within 24 hr of hatching, their heads frozen to $-20^\circ$C, then thawed, before dissection in TCT. Retinas were added to 1 ml of homogenizing buffer (0.05% Brij, 10 mM CaCl$_2$, 0.15 M NaCl, and 0.02 M phenylmethylsulfonyl fluoride (PM SF) in 0.025 M Tris, pH 7.5) and homogenized by 20 strokes in a homogenizer. Crude lysates were spun at 10,000 x g for 10 min to remove gross debris, the resulting pellets discarded, and the supernatant saved as “retinal cell extracts.” These were aliquotted and stored at $-80^\circ$C.

Protein content was determined (Bradford 1976), and equal amounts of protein were electrophoresed. SDS-PAGE was performed on a 5% stack/8% resolving gel in a Bio-Rad (Hercules, CA) mini-gel unit. A total of 5 x sample buffer (2% SDS, 25% glycerol, 60 mM Tris-HCl, pH 6.8, and 0.1% bromophenol blue) was added 1:4 to each sample, and then each was heated at 95$^\circ$C for 2 min before loading. Protein was transferred from the gel to a nitrocellulose membrane using a Bio-Rad semidry apparatus at 0.05 amps. Membranes were blocked overnight in the refrigerator in 3% BSA for 15 min. Blocking solution was replaced with TBS, and the membranes blocked and washed as described above.

For histochemistry, pieces of retina of various ages were fixed in 2% glutaraldehyde in 0.08 M phosphate buffer for 1 hr (Sheffield and Moscona 1970). They were then dehydrated and embedded in epon-araldite. Sections approximately 1$\mu$m thick were cut with a Porter Blum MT-2 microtome and mounted on glass slides. The slides were briefly heated to 90C after the addition of the sections.

Embedding material was removed by incubation in sodium ethoxide (prepared as a saturated solution of sodium hydroxide in absolute ethanol) for 15 min, rinsed in 100% ethanol, and the ethanol allowed to evaporate. Slides were then dipped briefly in TBS, then incubated in a blocking solution of 3% BSA for 15 min. Blocking solution was removed and a concentrated solution of avidin-peroxidase (Vector Elite ABC; 10 $\mu$l Reagent A, 10 $\mu$l Reagent B, and 1 $\mu$l Tween-20 per 1 ml Tris) was now added to the slides. Slides were incubated overnight at room temperature (RT) (refrigeration/agitation showed no effect). The following morning they were rinsed in Tris three times for 5 min. Slides were then incubated for 3 hr at RT with a sensitive peroxidase substrate, True Blue (Kirkegard & Perry Laboratories; Gaithersburg, M D). Slides were rinsed in ddH$_2$O, dehydrated, and mounted with Histoclad. Controls were treated exactly as above, except that overnight incubation was in Tris lacking the avidin-peroxidase reagent. Video images were digitized using the Snappy image capture system. Images were printed with a Fargo printer in dye-sublimation mode.

Results

An initial set of experiments confirmed the existence of the biotinylated carboxylases in the developing chick retina. These enzymes were detected specifically using the avidin–biotin interaction. Quantification indicated that the relative amounts steadily decreased with embryonic age. Figure 1 shows a Western blot of an age series of retinal cell extracts separated by SDS-PAGE, transferred to a nitrocellulose membrane, labeled with avidin-peroxidase, and developed with a treatment in 0.01 mg/ml avidin, three 5-min washes in Tris-buffered saline, pH 7.4 (TBS), a 15-min treatment in 0.001 mg/ml biotin, and three 5-min washes in TBS, before the normal avidin-peroxidase incubation. This strategy was necessitated by the structure of avidin and the nature of the avidin-peroxidase reagent employed (Vector Elite ABC; Vector Labs). Avidin contains four biotin binding sites (Green 1975). The avidin-peroxidase reagent actually consists of separate solutions of avidin– and biotin–peroxidase, which are mixed just before use. Incubation in biotin after the avidin blocking steps fills the remaining biotin binding sites on the blocking avidin, eliminating the possibility that unbound biotin–peroxidase in the ABC reagent binds and creates a false-positive signal. A second was incubated with avidin–peroxidase in lieu of avidin–peroxidase. A third was treated with Tris only. The fourth was treated exactly as the age-series blots above. After washing in TBS, the four pieces of the blot were incubated with the fluorescent substrate, re-assembled, and exposed to film, scanned, and quantified as above.

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fluorescent peroxidase substrate. The figure shows that avidin binding protein bands, presumably biotinylated proteins, are present in retinal cells of chickens of all ages from embryonic Day 8 (E8) to immediate post-hatch (PH). The approximate molecular masses of the bands are 130 and 70 kD. These are similar to those of the biotinylated subunits of multi-subunit enzymes previously localized to mitochondria (Chandler and Ballard 1986).

To quantify the age-dependent changes in the levels of the 130- and 70-kD avidin binding proteins, three sets of extracts from each age retina were collected and three Western blots were performed on each sample. Films were scanned by laser densitometry and quantified. Levels of each avidin binding protein, expressed in relative units, were averaged from all trials. Figure 2 shows the steady age-wise decrease of the 130-kD avidin binding protein. Over the course of the approximately 12 days from E8 to PH, the decrease is roughly 50%. The same effect is observed on the 70-kD avidin binding doublet, more pronounced in this case; the decrease over the same time period is greater than 80%.

The specificity of the interaction between avidin and the 70- and 130-kD proteins detected in retinal cell extracts was demonstrated by electrophoresing duplicate samples and transferring them to a nitrocellulose membrane. The membrane was then cut into pieces and subjected to several different treatments. One portion was preincubated with unlabeled avidin, followed by biotin (see Materials and Methods for further explanation), and then processed normally. A second was incubated with biotin-conjugated peroxidase in lieu of avidin-peroxidase. A third was incubated with TBS only. The fourth was treated exactly as the age-series blots above. After washing in TBS, the four pieces of the blot were incubated with the fluorescent substrate, reassembled, exposed to film, and scanned and quantified as above. The result is shown in Figure 3.

The specificity of the avidin reaction with retinal proteins was further tested by incubating with biotin-peroxidase (Figure 3, Lane 2) and substrate only (Figure 3, Lane 4) detected no proteins in the sample, indicating that the findings in Figure 1 do not result from nonspecific binding or endogenous peroxidase.

These results therefore suggested that we were indeed able to detect the biotinylated carboxylases of the mitochondrial matrix with avidin-peroxidase. A

Figure 1 Western blot of an age series of retinal cell extracts probed with avidin-peroxidase. Equal amounts of protein were loaded into each lane. Samples are, from left, embryonic Days 8, 10, 12, 14, 16, 18, and immediate post hatch.

Figure 2 Quantitation of biotinylated proteins in embryonic retina of different ages. The black bars represent the 130-kD band; white bars represent the 70-kD material. From each of three sets of retinal cell extracts, three experiments of the type shown in Figure 1 were performed. Resultant films were scanned with a laser densitometer and the relative intensity values were normalized between experiments and averaged. Units are arbitrary and error bars are shown.

Figure 3 Control for the specificity of the avidin reaction with retinal proteins. All lanes were loaded with equal amounts of the same E12 extract. Lane 1 was processed normally; Lane 2 was incubated with biotin-peroxidase in lieu of avidin-peroxidase; Lane 3 was blocked with unlabeled avidin before normal treatment; Lane 4 was incubated with fluorescent peroxidase substrate only.
complementary set of experiments, in which we sought to examine the morphological localization of avidin binding activity in tissue sections, was carried out. The results are shown in Figure 4. In the E8 retina, avidin binding activity is present and is dispersed throughout the tissue, although it is somewhat more concentrated in the vitreal and choroidal regions, i.e., the edges of the retina. This result is consistent with the relatively uniform, as yet unstratified morphology of the tissue at this stage of development. This can be seen in the adjacent Toluidine Blue-stained section of the E8 retina.

After 12 days of development (E12), the retina begins to take on its characteristic layered morphology. The distribution of avidin binding activity has also become striated; staining is concentrated in several bands corresponding to six specific regions within the tissue. From choroidal to vitreal, staining is seen in the areas containing the photoreceptor cell ellipsoids, the apical ends of the Müller cells, and on either side of the developing inner and outer plexiform layers.

With continued development (E14), staining around the plexiform layers gradually fades. In the post-hatch animal (PH), no staining whatever is visible in these regions. At the same time, avidin binding in the photoreceptors and Müller cells is readily visible in E14 and in PH.

Control experiments, in which sectioned retina was incubated with vehicle (Tris lacking the avidin-peroxidase reagent) followed by substrate, showed little if any staining. These data are not shown, because they are essentially blank panels. Likewise, sections of retina blocked by preincubation with unconjugated avidin, biotin, then substrate, and those incubated with biotin-peroxidase and substrate were without staining in all cases.

Discussion

Working with yeast, Louis Pasteur (1861) first described the inhibition of fermentation by oxygen. Warburg (1926) and M eyerhoff (1930) found a similar inhibitory effect of oxygen on the production of lactate in most tissues. The work of these authors provides the basis for the description of two means of energy production (metabolism) available to tissues: one that produces lactate and is inhibited by oxygen (glycolytic metabolism) and one that produces no lactate but depends on oxygen (oxidative metabolism). In a few tissues, however, this paradigm breaks down. Warburg et al. (1924) described several tissue-specific instances of “aerobic glycolysis,” i.e., the production of lactate in the presence of oxygen. One of the tissues exhibiting this type of metabolism was the retina of warm-blooded vertebrates.

The interesting metabolic properties of the retina have been known for some time. The avian retina, in particular, has been noted for its uniquely high rate of glycolytic activity. Enzyme histochemistry and immunohistochemistry directed at components of mitochondria, as well as direct observation by electron microscopy, have correlated this characteristic with the dearth of mitochondria present in the inner portion of the fully formed tissue (Krebs 1972; Buono and Sheffield 1991a). Previous work in our lab used the same techniques to describe a progressive loss of mitochondria from the developing chick retina (Buono and Sheffield 1991a). The result is a tissue that is, except for the photoreceptors, mostly devoid of oxidative capabilities, and therefore highly dependent on the glycolytic manner of metabolism. We showed that as the retina matures in the absence of a vascular supply, the lactate dehydrogenase isoform balance shifts from predominantly “H” (aerobic) to the “L” isoform (anaerobic) (Buono and Sheffield 1991b).

Several biotinylated enzymes—enzymes in which biotin is a covalently bound co-factor—present a novel avenue by which to further examine the fate of mitochondria during this process. Pyruvate carboxylase, methyl crotynyl-CoA carboxylase, and propionyl-CoA carboxylase are large multi-subunit proteins which have previously been localized to the mitochondrial matrix. Here, we have taken advantage of this fact, as well as the high-affinity binding between biotin and the glycoprotein avidin, to develop a relatively simple method of mitochondrial localization. The effort had its genesis as a result of “artifactual” bands of 70 and 130 kD, which appeared on control blots of retinal extracts (no antibody) for experiments employing biotinylated antibodies and avidin-peroxidase detection (H. Peng, personal communication). Although conventional peroxidase substrates based on diaminobenzidine did not produce detectable stain on sections, we were able to detect the biotinylated proteins in tissue sections with True Blue, an ultrasensitive peroxidase substrate.

Our results showed biotinylated proteins to be present in retinal cell homogenates. Western blot anal-
ysis (Figure 1) showed that the proteins were of two sizes, approximately 130 and 70 kD. These are the approximate sizes of the biotinylated subunits of the biotin-containing carboxylases of the mitochondrial matrix, as reported by others in mammalian tissues (Moss and Lane 1971; Chandler and Ballard 1986). A database search indicates that these enzymes are highly conserved in mammals and yeast. We would therefore expect little difference in avian species. Control experiments (Figure 3) suggest that the 130- and 70-kD proteins were detected on the basis of a specific biotin–avidin interaction rather than nonspecific binding (Figure 3, Lane 2) or endogenous peroxidase activity (Figure 3, Lane 4). Most importantly, for the developmental time period considered, E8 to PH, the quantity of both biotinylated proteins decreased markedly, although at different rates, suggesting a progressive loss of these enzymes, their essential co-factor, and/or mitochondria (Figure 2). This result is in agreement with the quantitative histochemistry and immunostaining for other mitochondrial enzymes, as well as the ultrastructural morphology previously reported by our lab (Buono and Sheffield 1991a).

When applied to tissue sections, avidin-based staining was also consistent with our earlier results (Figure 4). Although initially dispersed throughout the tissue (E8), endogenous biotin, and presumably mitochondria, segregate into several distinct regions of the increasingly stratified retina. In the mature retina, biotin–mitochondria is present in only two well-defined regions of the outer retina, corresponding to the photoreceptor cell ellipsoids and the apical feet of the Müller glia. This final pattern of staining, as well as the progressive loss of staining through development that leads to it, is similar to that previously seen in our lab using conventional methods of mitochondrial visualization.

Explanations for the lack of mitochondria in the mature inner retina are varied. One can consider its increasingly anoxic milieu, and draw analogy to the work of Ephrussi and Slominski (1950) in yeast. When grown anaerobically, the yeast mitochondria condense and lose many of the enzymes integral to oxidative metabolism. After subsequent addition of oxygen, these “pro-mitochondria” take on a normal morphology and again express the typical array of enzymes. Others have shown the regulatory properties of oxygen with regard to synthesis of molecules involved in metabolism (Guarente and Mason 1983; Trueblood et al. 1988). In the same vein, several groups have correlated the distribution of mitochondrial enzymes in the layered retina with its species-specific pattern of vascularization, i.e., oxygen supply (Hotta and Kato 1989; Buono and Sheffield 1991a).

On the other hand, the problem can be considered at an organismic level, as it is by Krebs, who correlates the avian inner retina, avascular and nearly devoid of mitochondria, with the acute eyesight of birds. Lack of blood supply and mitochondria are a means to an end, increasing the transparency of the retina and allowing better acuity then would otherwise be possible. Indeed, high-acuity regions of vascular retinas are also avascular. Finally, simple explanations, such as exclusion of mitochondria on the basis of size from the small-diameter cell processes of which the inner retina largely consists, have also been suggested (Buono and Sheffield 1991a). For those portions of the retina lacking mitochondria, it has been suggested that glycolysis may provide energy in vivo. This hypothesis has its problems, however. Constraints on glucose supply are likely to be similar to those on oxygen. The missing piece of the puzzle, a transport system for metabolites to the inner retina, or some arrangement of shared metabolism between cells in the retina, remains to be discovered.

Our work here provides another indication that large portions of the inner avian retina lack active mitochondria and therefore oxidative capability. It also describes the developmental changes that lead to this condition. This is achieved using labeled avidin, a novel approach to mitochondrial localization.

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