Cellular and Molecular Neurobiology, Vol. 1, No 4, 1981

Effect of Riboflavin on Monoamine Oxidase Activity in Cultured Neuroblastoma Cells

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Received July 16, 1981; accepted September 9, 1981

Monoamine oxidase (MAO) depends on a covalently attached FAD cofactor for activity. Activity is depressed in mouse neuroblastoma cells (N1E-115) grown in synthetic N2 medium lacking riboflavin. MAO activity in depleted cells is stimulated by added riboflavin, and this recovery is blocked by inhibitors of RNA and protein synthesis, and not by an inhibitor of protein glycosylation. Recovery from riboflavin depletion appears to depend upon new RNA and protein synthesis, and not on the addition of FAD cofactor to an inactive MAO precursor.

KEY WORDS: riboflavin; iron; neuroblastoma; monoamine oxidase; cell culture.

INTRODUCTION

Monoamine oxidase (MAO) (monoamine:O_2 oxidoreductase; EC 1.4.3.4) degradatively deaminates biogenic amines throughout the body. This enzyme is an integral protein in the outer mitochondrial membrane (Sottocasa *et al.*, 1967). It is thought to be a dimeric enzyme containing two polypeptide chains of similiar molecular weight, approximately 60,000, only one of which bears a covalently attached FAD cofactor (Oreland *et al.*, 1973: Salach, 1979). Although early studies indicated that the enzyme might contain and depend on iron for activity (Youdim, 1976; Salach, 1979), recent studies show that highly purified, active MAO molecules do not contain iron (Weyler and Salach, 1981).

When animals are starved for riboflavin or iron there is a gradual decrease in MAO activity levels by 15–85% over a period of 18 to 85 days (Kim and Lambooy, 1978; Kwatra and Sourkes, 1980; Youdim and Green, 1978). It is surprising that under conditions of severe riboflavin deficiency, MAO activity is not completely

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abolished; possibly riboflavin molecules required for activity are sequestered and reused under these conditions. Since iron is not necessary for activity of the purified enzyme, it is not clear why iron deficiency *in vivo* results in a loss of activity. Possibly iron is required for posttranslational processing of MAO precursor molecules, integrity of the outer mitochondrial membrane, or some other metabolic process which indirectly affects MAO activity.

A number of mammalian enzymes require a covalently attached FAD cofactor for activity. Four of these are located in the mitochondria—succinate dehydrogenase, sarcosine dehydrogenase, monoamine oxidase, and an as yet unidentified flavoprotein (Addison and McCormick, 1978; Sato *et al.*, 1977). No information is available as to the means, presumably enzymatic, whereby the flavin cofactor is attached to these proteins or when and where this event takes place during their synthesis and insertion into the mitochondria. Incorporation of [^{14}C]-riboflavin into mitochondrial flavoproteins *in vivo* continues for several hours in the presence of an inhibitor of protein synthesis, suggesting that flavin incorporation can occur after translation of the apoenzyme is completed (Martinez and McCauley, 1977). In contrast, recent studies by Hamm and Decker (1978) have shown that attachment of the FAD cofactor to 6-hydroxy-D-nicotine oxidase in *Arthrobacter oxidans* occurs during translation when synthesis of the polypeptide is only about half-completed.

In the present study we used a cell culture system to gain insight into the requirements of iron and riboflavin for synthesis of active MAO molecules. Mouse neuroblastoma cells of the adrenergic line N1E-115, which contain only the A type of MAO activity (Donnelley *et al.*, 1976), were used. Cells were grown in synthetic N2 medium (Bottenstein and Sato, 1979) with or without riboflavin, iron, and other compounds. Changes in MAO activity were followed over time in culture. [^{14}C]Riboflavin-labeled mitochondrial proteins were analyzed by SDS–polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Cell Culture. Murine neuroblastoma line N1E-115 was used between 15 and 30 subcultures after isolation as a single cell clone (Amano *et al.*, 1972). Cells were maintained on synthetic N2 medium (Bottenstein and Sato, 1979) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Stock flasks (75 cm^2, Costar) were fed at 3- to 4-day intervals and subcultured at a ratio of 1:10 following resuspension by trituration. For experiments, cells were plated onto dishes (60 or 100 mm, Costar) coated with poly-D-lysine (McKeehan and Ham, 1976) and fed N2 medium, modified as indicated, at 1- or 2-day intervals.

Removal of Iron from Transferrin. For some experiments transferrin (Sigma) was treated so as to remove bound iron as described (Pollack *et al.*, 1977). Transferrin was dissolved in 0.1*M* Hepes, pH 7.4, to a final concentration of 0.1 m*M* and dialyzed for 24 hr at 4°C against 100 vol of 0.1 *M* Hepes, pH 7.4, containing 0.5 m*M* desferrioxamine (Desteral, Ciba) and 1 m*M* pyrophosphate.

Harvesting Cells and Preparation of Mitochondrial Fractions. Monolayers were rinsed twice with isotonic phosphate-buffered saline and scraped off dishes in a

small volume of the same (Hawkins and Breakefield, 1978). Cells were collected by centrifugation at 7000*g* for 1 min, and cell pellets were stored at −65°C.

A crude mitochondrial fraction was prepared from homogenized cells by differential centrifugation (Costa and Breakefield, 1979). In this procedure a mitochondrial pellet is collected by centrifugation at 10,000*g* for 20 min. In some experiments both the supernatant and the mitochondrial pellet were tested for activity.

Assays. For MAO determinations, samples were resuspended in 10 m*M* phosphate buffer, pH 7.4 to give a final concentration of 1–5 μg protein/μl and sonicated three times for 10 sec on ice at 100 W (Bronwill microprobe). Enzme assays were carried out using [^{3}H]tryptamine by a modification of the method of Wurtman and Axelrod (1963), as described (Costa *et al.*, 1980). Reactions were carried out in 1-dram shell vials; following acidification, scintillation fluid was added directly to the vial. After shaking for 3 min to extract deaminated products into the toluene layer, the entire vial was counted in a liquid scintillation spectrometer (Edelstein and Breakefield, 1981). Homogenates were assayed in triplicate at two protein concentrations within the range of linearity with respect to time and protein.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. Iron determinations were performed by Dr. Peter Jatlow at Yale using flameless atomic absorption (Olson and Hamelin, 1969).

Labeling of MAO and Flavoproteins. Monoamine oxidase was specifically labeled with [^{3}H]-pargyline using crude mitochondrial preparations from N1E-115 cells grown in the presence of riboflavin (Pintar *et al.*, 1979). To label flavoproteins, cultures on 150-mm dishes were maintained on N2 lacking riboflavin for 5 days, then fed N2 containing 0.22 mg/liter D-[2-^{14}C]ribloflavin (sp act 60.6 mCi/mmol; Amersham). After 48 hr of growth in the latter medium, a crude mitochondrial fraction was prepared.

Labeled mitochondrial proteins were solubilized and analyzed by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (Cawthon *et al.*, 1981). Gel were prepared for autoradiography by incubation with En^{3}Hance (NEN), drying, and exposure to preflashed Kodak X-omatic R film for 2 weeks at −65°C. To quantitate radioactivity gels were dried and cut into 0.5-cm strips, and radioactivity in the gel slices was determined by a modification of the method of Horvitz (1973) as recommended by New England Nuclear.

RESULTS

Effect of Riboflavin and Iron Depletion and Riboflavin Resupplementation on MAO Activity

Cultures at 60–80% confluency were rinsed once with N2 medium lacking riboflavin and/or iron and maintained in the same medium with daily feedings for up to 10 days. When cells were grown in medium lacking riboflavin, MAO activity began to decrease after 2 days of deprivation and reached a low point of 50% of control levels after 5 days, with no further decrease over 10 days (Fig. 1A). After 6 days without riboflavin some cell death began to occur, as noted by the presence of round, floating cells. Parallel cultures fed with N2 containing riboflavin, at the normal concentration

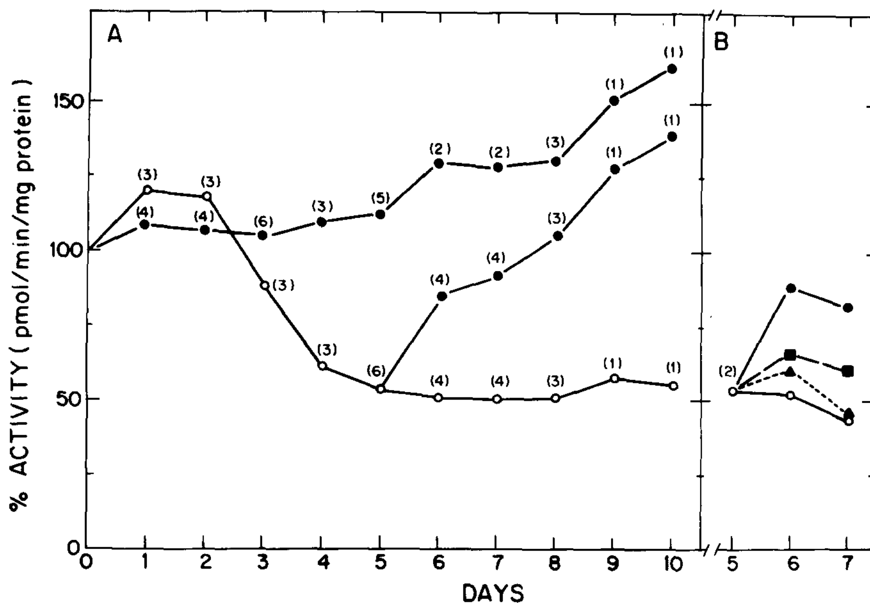

Fig. 1. MAO activity in cells grown with and without riboflavin. (A) Parallel dishes of N1E-115 were grown in the presence (●) or absence (○) of riboflavin. MAO activity was measured in cell homogenates; 100% represents an average activity of 156 pmol/min/mg protein ± 34 SD (N = 10). Each point is the average of normalized values from (N) number of separate experiments. (B) Cells were grown in medium without riboflavin for 5 days, then in medium with (filled symbols) or without (open symbols) riboflavin for 2 days. To some dishes either 0.35 μM cycloheximide (▲) or 50 μM camptothecin (■) was added with the riboflavin.

of 0.58 μM, showed an increase in specific MAO activity and in cell density over 10 days (Fig. 1A). When iron was omitted from the medium over 9 days, there was no change in MAO activity as compared to control levels. This was observed whether or not transferrin in the medium had been treated to remove bound iron. Levels of iron in homogenates from depleted cells were only 10–30% lower than in control cells; the latter had 87 ng iron/mg cell protein. When both iron and riboflavin were lacking from the medium, MAO activity was similar to that observed for medium lacking only riboflavin.

When cultures were refed with medium containing riboflavin (0.58 μM) after 5 days of riboflavin deprivation, MAO activity levels rose by 30–40% within 24 hr and continued to increase, approaching control levels after 3 days (Fig. 1A). The rate and extent of recovery of MAO activity were not affected by the addition of 10 times as much riboflavin (5.8 μM), tunicamycin (5 μg/ml), or dexamethasone (50 nM) or by the absence of iron (data not shown). Further, fractionation studies showed that essentially all MAO activity was found in the crude mitochondrial fraction and not in the cytoplasmic fraction following riboflavin depletion and 5 and 24 hr after riboflavin resupplementation (data not shown).

Dependence of Recovery of MAO Activity on Protein Synthesis

Recovery of MAO activity during the first 2 days following riboflavin resupplementation was blocked 80% by an inhibitor of protein synthesis, cycloheximide (Fig.

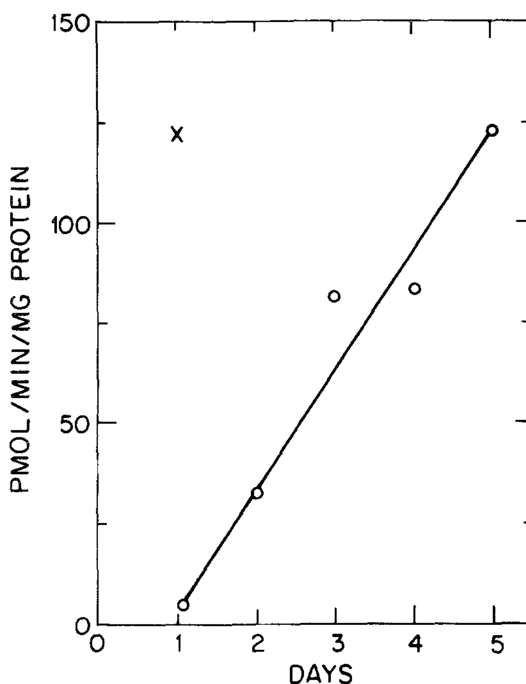

Fig. 2. Recovery of MAO activity after inhibition with clorgyline. On day 1, cultures of N1E-115 at 90% confluency were incubated with 10 n*M* clorgyline for 1 hr at 37°C, then rinsed twice with drug-free medium and incubated in the same for 5 days. Cells were harvested at daily intervals and MAO was assayed in cell homogenates. Values are given prior to (×) and following (O) inhibition.

1B), at a concentration which inhibited the incorporation of [^{14}C]leucine into TCA-precipitable protein by 80%. Recovery was blocked 59% by an RNA-synthesis inhibitor, camptothecin (Fig. 1B), at a concentration which inhibited 83% of the [^{14}C]uridine incorporation into TCA-precipitable RNA. The action of inhibitors on protein and RNA synthesis was tested over 24 and 48 hr in nondepleted cultures at cell densities comparable to that in depleted cultures. Cell viability, assessed by trypan blue exclusion, was not affected by these inhibitors under these conditions.

The rate of recovery of MAO activity upon riboflavin resupplementation was essentially identical to the rate of recovery observed in riboflavin-fed cells in N2 medium after the irreversible inactivation of MAO molecules with the MAO inhibitor clorgyline (Fig. 2). The latter reflects the rate of synthesis of new active MAO molecules.

Incorporation of [^{14}C]-riboflavin into Mitochondrial Flavoproteins After Riboflavin Depletion

Following 5 days of riboflavin depletion, cultures were supplemented with [^{14}C]riboflavin to give a final concentration comparable to that in N2 medium. After 2 days of labeling, cells were harvested and a crude mitochondrial fraction was prepared

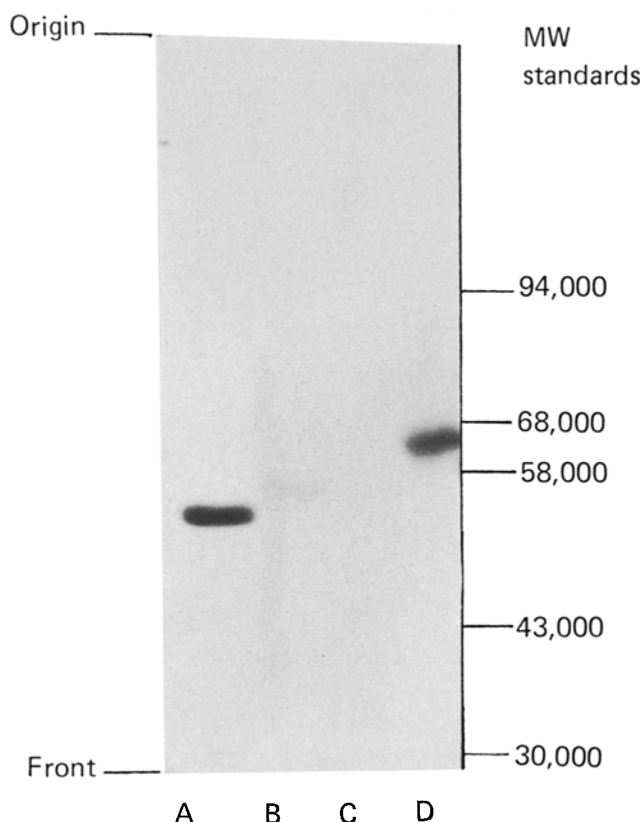

Fig. 3. Autoradiogram SDS-PAG of [^{14}C]-riboflavin- and [^{3}H]-pargyline-labeled mitochondrial proteins. Crude mitochondrial fractions were prepared from rat hepatoma line MH_1C_1 (A), mouse neuroblastoma line N1E-115 (B and D), and mouse neuroblastoma line N1E-115TG2 (C). Samples in lanes A, B, and C were labeled with [^{3}H]-pargyline as described (Cawthon *et al.*, 1981). The hepatoma sample was preincubated with 0.1 μM clorgyline to block MAO A sites prior to [^{3}H]-pargyline binding to MAO B sites. The sample in lane D is from N1E-115 cells grown without riboflavin for 5 days, then with [^{14}C]-riboflavin for 2 days.

from them. SDS–polyacryamide gel electrophoresis of mitochondrial proteins and autoradiography revealed a single labeled band of apparent molecular weight 68,000 (Fig. 3). This [^{14}C]riboflavin-labeled protein did not comigrate with [^{3}H]pargyline-labeled MAO and is presumed to be succinate dehydrogenase on the basis of its molecular weight. Apparently MAO represents a fraction of the [^{14}C]-riboflavin molecules too small to be revealed under these conditions.

DISCUSSION

Little is known about the cotranslational or posttranslational processing of MAO molecules. At some stage the flavin cofactor is attached, the two subunits become

associated, and the protein is inserted into the outer mitochondrial membrane. Studies also suggest that a sialic acid residue may be important for the activity of type A MAO, indicating that glycosylation of the molecule may occur (Houslay and Marchmont, 1980), and that iron is necessary for the processing and/or activity of MAO (Youdim, 1976).

Here, cultured mouse neuroblastoma cells were grown in synthetic medium lacking riboflavin and/or iron. Riboflavin depletion resulted in a substantial loss of MAO activity; it was not possible to obtain a complete loss of activity under these conditions due to cell death. Death presumably results from the loss of activity of other enzymes which use riboflavin as a cofactor. Monoamine oxidase activity itself does not appear to be necessary for cell viability in culture, as a variant line, N1E-II5TG2, which lacks MAO activity (derived from N1E-115; Breakefield *et al.*, 1976), grows at a rate comparable to N1E-115 in N2 medium.

When riboflavin is added back to riboflavin-depleted cells, MAO activity returns at a rate similar to that observed in cells recovering from irreversible inactivation of MAO molecules. In the latter case recovery depends on the synthesis of new MAO polypeptides (Erwin and Deitrich, 1971). The similiarity in rates of recovery suggests that the recovery of MAO activity in riboflavin-depleted cells depends on new protein synthesis. This observation is strengthened by the finding that riboflavin-mediated recovery is blocked almost completely by the inhibition of protein synthesis and blocked substantially by the inhibition of RNA synthesis.

The dependence of riboflavin-mediated recovery of MAO activity on new protein synthesis could be explained in a number of ways. It is possible that, as in the case of 6-hydroxy-D-nicotine oxidase (Hamm and Decker, 1978), the flavin cofactor is normally added onto the MAO polypeptide during translation. If this were the case, unavailability of the flavin cofactor during translation might result in the formation of flavin-less MAO polypeptides. Alternatively, it is possible that the flavin cofactor is normally added to MAO molecules after translation, before or after the subunits become associated and inserted into the mitochondrial membrane. Our findings indicate that following riboflavin deprivation there is not a pool of flavin-less MAO polypeptides to which FAD can be attached. This would be consistent with any of the following explanations: (1) Enzymes responsible for the synthesis of FAD, attachment of FAD to MAO, or processing of MAO may be decreased in amount during riboflavin deprivation and may depend on new protein synthesis to regain their activity. (2) Due to conformational restrictions or further processing or insertion events, the FAD cofactor may be able to be attached to the MAO polypeptide only during, and not after, translation. (3) Monoamine oxidase polypeptides lacking FAD may be rapidly degraded. The resolution of these possibilities will depend on the availability of antibodies which can recognize precursor as well as active forms of MAO.

The question of the role of iron in maintaining MAO activity *in vivo* is still not clear. We were unable to deplete cells of iron to a substantial extent by omitting iron and including iron-free transferrin in the medium. Up to 30% depletion of cellular iron did not affect levels of MAO activity. Although iron is not a component of active MAO molecules, it may be necessary for the activiy of other enzymes involved in the processing of MAO molecules to an active form.

SUMMARY

(1) When mouse neuroblastoma cells of line N1E-115 were grown on synthetic N2 medium lacking riboflavin, monoamine oxidase (MAO) activity decreased by 50% over a period of 5 days.

(2) Resupplementation of depleted cells with riboflavin resulted in an increase in MAO activity, which paralleled the rate of increase in nondepleted cells following a brief treatment with an irreversible inhibitor of MAO. The recovery of activity in response to riboflavin was prevented by inhibitors of RNA and protein synthesis, camptothecin and cycloheximide, respectively.

(3) Recovery was not affected by an increased amount of riboflavin or the presence of tunicamycin, an inhibitor of protein glycosylation.

(4) All MAO activity during depletion and recovery was associated with the mitochondrial fraction from cells. Analysis of mitochondrial proteins labeled with [^{14}C]riboflavin during recovery by SDS–polyacrylamide gel electrophoresis revealed a single labeled protein species of apparent molecular weight 68,000. This labeled protein is presumed to be succinate dehydrogenase; other mitochondrial proteins containing a covalently attached flavin cofactor, including MAO, were not labeled to a sufficient extent to be detected under these conditions.

(5) It was not possible to evaluate the involvement of iron in MAO activity as the omission of iron and inclusion of iron-depleted transferrin in the medium did not result in a substantial reduction of iron in the cells.

(6) The major insight that can be drawn from these studies is that recovery of MAO activity following riboflavin depletion appears to depend on new protein synthesis, and thus an inactive MAO precursor molecule does not appear to accumulate under these conditions.

ACKNOWLEDGEMENTS

We thank Susan Edelstein for her help. This grant was supported by NIH grant GM20124.

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