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RHODAMINE 123 INHIBITS IMPORT OF RAT LIVER MITOCHONDRIAL TRANSHYDROGENASE*

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Rhodamine 123, a laser dye, has been demonstrated to inhibit import of the precursor to pyridine dinucleotide transhydrogenase into mitochondria in rat liver cells. When rat hepatocytes were labeled with ^{35}[S] methionine in the presence of 0.4 mM rhodamine 123, the precursor to transhydrogenase was found to have a half-life in the cytoplasm of 15 minutes as opposed to a half-life of 1-2 minutes when cells were radiolabeled in the absence of the dye. To clarify the mechanism of import inhibition, studies were initiated to assess the effect of rhodamine 123 on mitochondrial respiration. Upon addition of the dye to a mitochondrial suspension, respiration was initially enhanced, then inhibited. The inability of FCCP, a classical uncoupler, to enhance respiration during the inhibitory phase suggests that rhodamine 123 is primarily inhibiting respiration through the electron transport system rather than through the ATPase. These results suggest that rhodamine 123 may inhibit import of the transhydrogenase precursor into mitochondria by disrupting components in the mitochondrial membrane necessary for efficient import.

Pyridine dinucleotide transhydrogenase is an integral mitochondrial inner membrane enzyme that couples the reversible transfer of a hydride ion between matrix NADPH and NAD^+ to vectorial proton translocation from the matrix to the cytosol (1,2). The enzyme has been previously reported to be synthesized initially as a larger molecular weight precursor in the cytosol (3). The newly synthesized precursor is then translocated into the mitochondria with a half-life of 1-2 minutes in the cytoplasm and processed to the mature enzyme by a matrix localized protease.

Rhodamine 123, a laser dye, has been previously shown to inhibit import of precursors to carbamyl phosphate synthetase I and ornithine transcarbamylase into

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mitochondria of rat hepatocytes (4). Other studies have shown that this dye inhibits mitochondrial respiration either by inhibiting the FoF_1 ATPase or possibly electron transport reactions (5,6). Data from Abou-Khalil *et al.* (7) suggest that rhodamine might be acting through an uncoupler-like mechanism. These reports prompted us to investigate how rhodamine 123 may affect the import of transhydrogenase precursor into mitochondria of rat hepatocytes. Also, since respiration is closely coupled to many mitochondrial processes, we thought that by correlating results of the import experiments with those from studies designed to determine the effect of rhodamine 123 on mitochondrial respiration, insight into how this dye inhibits import of the transhydrogenase precursor might be obtained.

Materials and Methods

Materials - Male Sprague-Dawley rats were obtained from Harland Sprague Dawley (Indianapolis, IN). Dialyzed fetal calf serum was from Gibco (Grand Island, NY). Carbonylcyanidetrifluoromethoxyphenylhydrazone (FCCP) was purchased from Pierce Scientific (Rockford, IL). Rhodamine 123 and acrylamide were obtained from Eastman Kodak (Rochester, NY). [^{35}S] methionine was from DuPont-New England Nuclear (Boston, MA). Tris, Hepes, TES, Tricine, cycloheximide, digitonin, 1,10 phenanthroline, antipain, leupeptin, phenylmethylsulfonylfluoride (PMSF), ADP, malate and amino acids were obtained from Sigma Biochemicals (St. Louis, MO).

Methods - Hepatocytes were prepared from male Sprague-Dawley rats (150-250 grams) by the procedure of Seglen (8). Cells were diluted to a concentration of $4x10^7$ cells/ml with suspension buffer composed of 68 mM NaCl, 1.2 mM $CaCl_2$, 30 mM Hepes, 5.4 mM KCl, 1 mM $MgCl_2$, 1.1 mM KH_2PO_4, 30 mM TES, 36 mM Tricine, 10 mM dextrose (pH 7.6). The hepatocyte suspension (0.2-0.5 ml) was made to 5% (v/v) with fetal calf serum and to 0.1 mM with the 19 common amino acids, lacking methionine. Some incubations contained 0.4 mM rhodamine 123. Radiolabeling of the cells was initiated by addition of ^{35}[S] methionine (1000 Ci/mmole) to 600 µCi/ml. Cells were labeled for 10 minutes at 37°C with shaking at 250 RPM in a new Brunswick Scientific water bath. In pulse-chase experiments, hepatocyte suspensions were made to 1 mM with unlabeled methionine and 1 mM cycloheximide after the pulse.

Following the pulse, or at various times during the chase, 100 µl aliquots were taken for analysis by immunoprecipitation. In some cases, labeled hepatocytes were first fractionated into soluble and membranous components using the following digitonin extraction procedure. The labeled cell suspension (100 µl) was added to 0.9 ml of a buffer containing 150 mM NaCl, 4 mM EDTA, 2 mM 1,10-phenanthroline, 20 µg/ml antipain, 20 µg/ml leupeptin, 1 mM PMSF, 30 µM FCCP, 1 mg/ml digitonin, and 40 mM Tris-HCl, pH 7.5. The mixture was incubated for 10 minutes at 4°C with occasional agitation. The digitonin-treated cell suspension was then diluted to 3 ml by addition of the same buffer lacking the detergent. The sample was centrifuged at 5,000 RPM for 5 minutes in a Beckman JA-20 rotor to separate the supernate from the pellet. To the supernate was added 30 µl of 20% (w/v) SDS and 15 µl of 20% (v/v) Triton X-100. The pellet ws solubilized in 0.4 ml of a buffer containing 150 mM NaCl, 5 mM EDTA, 2 mM 1,10 phenanthroline, 1 mM PMSF, 2% (w/v) SDS, 1% (v/v) Triton X-100, and 50 mM Tris-HCl, pH 7.5. The sample was then diluted to 3 ml with a buffer containing 150 mM NaCl, 5 mM EDTA, 2 mM 1,10 phenanthroline, 1

mM PMSF, and 50 mM Tris-HCl, pH 7.5. Both the particulate and soluble extracts were then centrifuged at 40,000 RPM for 30 minutes in a Beckman Ti-50 rotor. Transhydrogenase and its precursor were immunoprecipitated from each fraction and from whole cell extracts as previously described (3). The immunoprecipitates were analyzed by 5-15% SDS-polyacrylamide gel electrophoresis and fluorography. Rat liver mitochondria were prepared as described previously (9). Mitochondrial respiration was measured using a Clark oxygen electrode attached to a Gilson oxygraph. The 2.7 ml assay mixture contained mitochondria (0.8 mg protein), 5 mM $MgCl_2$, 100 mM KCl, 5 mM K_2HPO_4, 10 mM glutamate, and 5 mM malate, pH 7.5 (10). When indicated, 27 µl of 30 mM ADP, 1 mM FCCP, 100 mM KCN, or 4 mM rhodamine 123 were added to the reaction vessel.

Results and Discussion

To study the effect of rhodamine 123 on import of the transhydrogenase precursor into rat liver mitochondria, freshly prepared hepatocytes were labeled with [^{35}S] methionine in the presence or absence of 0.4 mM rhodamine 123 (Figure 1). At this concentration, the dye did not inhibit protein synthesis as determined by incorporation of radiolabel into protein. After a 10 minute pulse, cells were fractionated into soluble and particulate components and transhydrogenase was immunoprecipitated from each fraction, analyzed by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. When hepatocytes were labeled in the absence of rhodamine 123, nearly equal amounts of the precursor and the mature enzyme were found in the cytosol (lane 2) and the particulate fraction (lane 1), respectively. In contrast, with cells labeled in the presence of the dye the precursor

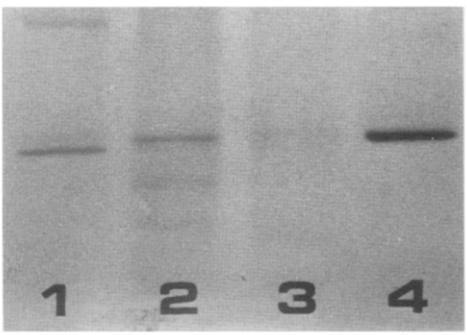

Figure 1. *Effect of rhodamine 123 on conversion of transhydrogenase precursor to the mature enzyme.* Hepatocytes were pulsed for 10 minutes with [^{35}S] methionine in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 0.4 mM rhodamine 123. Radiolabeled hepatocytes were fractionated into particulate (lanes 1 and 3) and soluble (lanes 2 and 4) components. Transhydrogenase was detergent extracted, immunoprecipitated, resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography as described under "Methods".

was found to accumlate in the cytosol (lane 4) with little mature enzyme appearing in the particulate fraction (lane 3). To determine the extent to which rhodamine 123 inhibited import of the transhydrogenase precursor, hepatocytes were labeled with [^{35}S] methionine in the presence of rhodamine 123 and chased following the addition of cycloheximide and unlabeled methionine. Aliquots were removed from the chase mixture at various times. Transhydrogenase and its precursor were then immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Under these conditions, the precursor was observed to have an apparent half-life of about 15 minutes (Figure 2). Since it has been previously reported that in the absence of rhodamine 123, the precursor has an apparent half-life of 1-2 minutes (3), it can be concluded that the dye effectively inhibits the import of transhydrogenase into mitochondria.

Study of protein translocation into mitochondria has been hampered by the lack of inhibitors of known specificity. Thus far, three classes of inhibitors have been found: (1) ionophores and protonophores which inhibit the import of many, but not all, mitochondrial protein precursors by depleting the electrochemical gradient across the inner membrane; (2) deuterohemin, a reversible inhibitor of the heme

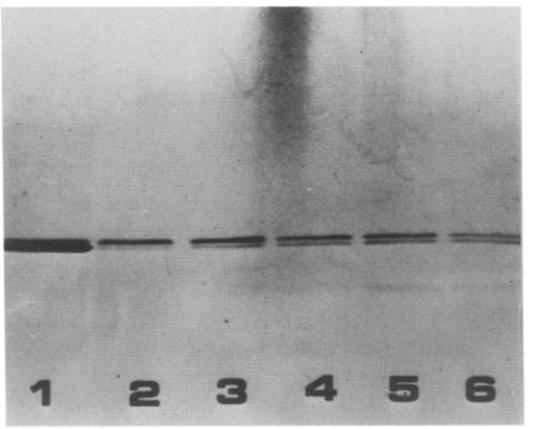

Figure 2. *Effect of rhodamine 123 on the uptake of transhydrogenase precursor into mitochondria.* Hepatocytes were pulsed with [^{35}S] methionine in the presence of 0.4 mM rhodamine 123 and chased in the presence of cycloheximide with unlabeled methionine. At various times, aliquots were removed, detergent-extracted, and immunoprecipitated. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1 represents an immunoprecipitate pulsed in the absence of rhodamine 123. Lanes 2-6 contain immunoprecipitates obtained after 0, 3, 6, 9, and 12 minutes of chase, respectively.

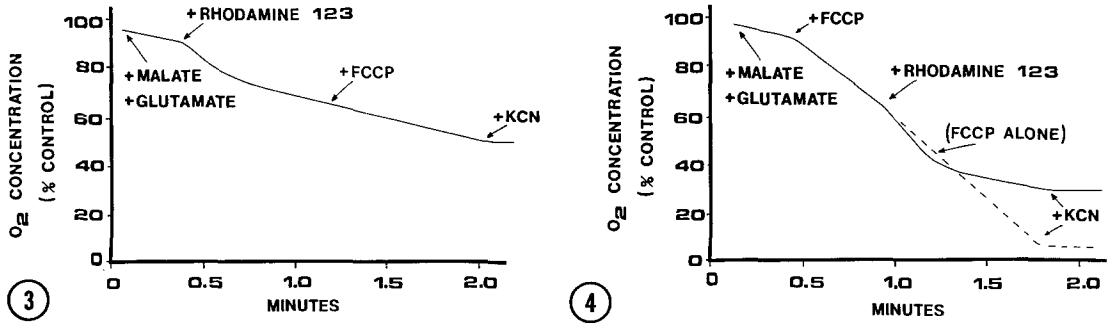

Figure 3. *Effect of rhodamine 123 on mitochondrial respiration.* Respiratory rate was measured using a standard Clark oxygen electrode. 100% control refers to an oxygen saturated solution. The 2.7 ml assay mixture (pH 7.5) contained 0.8 mg rat liver mitochondrial protein, 5 mM kCl, 5 mM k_2HPO_4, 10 mM glutamate, and 5 mM malate. Where indicated, 27 µl of the following was added: 4 mM rhodamine 123, 1 mM FCCP, and 100 mM KCN.

Figure 4. *Effect of rhodamine 123 on uncoupled mitochondrial respiration.* Respiration was measured as described in Figure 3.

attachment step which effectively inhibits the import of apocytochrome c; and (3) synthetic leader sequences which compete with the *in vitro*-synthesized precursor for binding to mitochondria (11). Since respiration is intimately linked to mitochondrial structure and function, it was thought that results from the studies on the effect of rhodamine 123 on mitochondrial respiration would give insight into how import of the transhydrogenase precursor was being inhibited. Such knowledge may make rhodamine 123 useful as a reagent for the study of protein import into mitochondria.

Initial studies were aimed at determining the effect of rhodamine 123 on mitochondrial respiration. It was found that respiration was initially enhanced by rhodamine 123 (40 µM) (Figure 3). Later this effect was gradually lost and the dye was found to block the known stimulatory effect of FCCP on respiration. While the respiratory enhancement brought about by the dye has been reported by other workers (5,6), the ability of rhodamine 123 to inhibit subsequent stimulation of respiration has not been previously reported. It is interesting to note that rhodamine 123 did not completely inhibit respiration, but rather limited its rate to approximately that of substrate level respiration (Figure 3). Thus, even upon addition of substrate (succinate or malate + glutamate), ADP, or FCCP, respiration would not be further enhanced during the inhibitory phase. Since respiration was measured at the level of cytochrome c oxidase, these results suggest that rhodamine 123 directly or indirectly affects the complexes of the mitochondrial electron

transport system. Additionally, the inability of FCCP to stimulate respiration demonstrates that respiratory inhibition by rhodamine 123 is not a consequence of inhibition of the ATPase (Figure 4).

It has been reported that rhodamine 6G, a more lipophilic structural analog to rhodamine 123, inhibits the import of methylmalonyl-CoA mutase into rat liver mitochondria (12). This compound is believed to bind to lipid and protein components of mitochondria (13). Both of these cationic compounds have been shown to have similar effects on mitochondrial respiration (6,13). While it may be possible that rhodamine 123 also binds to lipid because of its structural and functional similarity to rhodamine 6G, data by Johnson *et al.* (14) indicate that the dye does not bind to all lipid membranes, and also may be sequestered into the mitochondrial matrix. Since many of the respiratory complexes have a lipid requirement for their function, *e.g.* cytochrome oxidase for cardiolipin (15), it is quite conceivable that rhodamine 123 may alter membrane structure by binding to lipid. Thus, it may affect indirectly the respiratory complexes as well as other membrane components necessary for efficient precursor import.

The next logical course of study would be to examine the interaction of rhodamine 123 with various liposome and proteoliposome preparations. Additionally, liposomes may be made such that they possess an electrochemical gradient. How rhodamine 123 may affect such a gradient can be determined and the results applied to more direct testing of the effect of this compound on mitochondrial structure and function.

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