Evidence for a Role of Tyrosyl Residues in Cell Membrane Permeability*

JOSEPH G. CORY

From the Department of Chemistry and the Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida

SUMMARY

In the presence of tyrosinase, red blood cells are lysed. Lysis follows an enzyme-mediated reaction, with the amount of lysis being proportional to the tyrosinase concentration. Cyanide, which inhibits tyrosinase, prevents lysis of the red blood cells by tyrosinase. Heat-denatured tyrosinase has no effect on red blood cells. Tyrosinase also exhibits a pronounced effect on the permeability of isolated rat diaphragms. Glucose uptake is inhibited 28 to 44% whereas glycine uptake is stimulated 50% by tyrosinase. Ehrlich ascites cells, in the presence of tyrosinase, show decreased viability as measured by eosin dye uptake. These data strongly suggest that the tyrosyl residues of the protein layers of the cell membrane are involved in the maintenance of cell membrane integrity.

The role of amino acid side chains of the protein layers of cell membranes has not been studied to any extent to determine if any of these groups are important in the control of membrane permeability. However, Riley and Lehninger (1) have shown that the sulfhydryl groups of mitochondrial membrane proteins are involved in mitochondrial swelling and contraction. The action of sulfhydryl reagents on the effect of red cell membrane permeability has recently been reviewed (2).

It is well established that the tyrosyl residues of some intact proteins are oxidized in the presence of purified preparations of tyrosinase (3-7). In some cases, oxidation of the tyrosyl groups of biologically active proteins results in the loss of biological activity (3, 7). In addition, Sizer, Brindley, and Wagley (8) reported that red blood cells are agglutinated in the presence of tyrosinase.

In this communication, evidence is presented to show that highly purified mushroom tyrosinase not only causes agglutination of red blood cells, but also causes lysis of these cells. It is also shown that the permeability of isolated rat diaphragms is affected by the presence of tyrosinase. Glucose uptake is inhibited while glycine uptake is markedly stimulated. Finally, Ehrlich ascites cells show decreased viability when incubated with tyrosinase.

METHODS

Red blood cells were obtained from heparinized blood of freshly killed rats. The red cells were washed with cold Ringer's solution and centrifuged. A stock suspension of red cells was made by mixing the cells from 1 ml of blood with 20 ml of Ringer's solution (9). Aliquots of this suspension were used for the studies. A typical reaction mixture consisted of red cells, Ringer's solution, and tyrosinase in phosphate buffer, pH 7, 0.05 M. The control mixture contained cells, Ringer's solution, and tyrosinase in phosphate buffer, pH 7, 0.05 M with no enzyme. The control mixture contained cells, Ringer's solution, and buffer in place of enzyme.

Red blood cell lysis was measured by removing aliquots at various times, centrifuging at high speed, and measuring the absorbance of the supernatant solution at either 400 or 540 nm. The reactions were carried out at room temperature (24°C). Rat diaphragms were removed from freshly killed animals, cut into hemidiaphragms, and washed with cold Ringer's solution. Glucose uptake was measured essentially by the procedure of Oyama, Hagen, and Grant (10) for insulin bioassay, except that glucose was measured by the Glucostat method (Worthington). Two hemidiaphragms were randomly chosen and placed in a 20-ml beaker containing 2 mg of glucose, 1 ml of Ringer's solution, and tyrosinase in a final volume of 3.0 ml. The controls contained buffer in place of tyrosinase. The beakers were shaken on a Dubnoff metabolic shaker for 90 min. Oxygen was passed into the chamber for the first 10 min of the incubation period. After the reaction period, the supernatant solutions were poured off, duplicate 0.2-ml aliquots were taken, and 1.0 ml of 1.8% Ba(OH)2·8H2O and 1.0 ml of 2.0% ZnSO4·7H2O were added to precipitate the protein in the samples. The mixtures were centrifuged and 1.0-ml samples of the protein-free supernatant were taken in duplicate for glucose determinations.

Ehrlich ascites cells were taken from mice 6 or 7 days after transplantation and washed with 0.9% NaCl. A 5% suspension by volume was used as the stock suspension. Viability of the ascites cells was measured by eosin dye uptake. Aliquots of the stock suspension were incubated with tyrosinase in 0.9% NaCl and phosphate buffer. Control samples contained no tyrosinase.

The mushroom tyrosinase (o-diphenol:oxygen oxidoreductase, EC 1.10.3.1) preparations used in these experiments were pre-

* This work was supported by Grant HE 08344 from the National Institutes of Health.
‡ Present address, Department of Chemistry, University of South Florida, Tampa, Florida 33620.
pared by the procedure of Frieden and Ottesen (11). Several preparations were isolated in this laboratory, while another preparation was a gift from Sigma.

The tyrosinase preparations were homogeneous in the ultracentrifuge and were free of detectable protease activity as measured previously (6). A tyrosinase enzyme unit is defined as that amount of enzyme which will cause a change in optical density of 0.001 unit per min when measured at 475 μA at a tyrosine concentration of 3 × 10⁻⁴ M in 0.066 M sodium phosphate buffer, pH 7.5, and at a temperature of 30°. This unit is equivalent to 0.036 Enzyme Commission units as defined by Fling, Horowitz, and Heinemann (12). The specific activities of the tyrosinase preparations used were 5,900 to 10,600 tyrosine units per mg of protein.

Glycine-2-¹⁴C uptake was measured by following the disappearance of glycine-2-¹⁴C from the incubation medium. The hemidiaphragms were prepared in the same manner as for the glucose uptake experiments. The incubation mixtures contained in a final volume of 3.1 ml, Ringer’s solution, 2 mg of glucose, phosphate buffer, 0.04 M, pH 7, and 0.5 μC of glycine-2-¹⁴C (130 mC per mmole). After incubation for 90 min the supernatant solutions were poured off and aliquots were taken for radioactivity measurements. The diaphragms were blotted dry, weighed, and frozen. The diaphragms were then pooled by groups, homogenized in 10% trichloroacetic acid, and the homogenate centrifuged. The supernatant solutions were removed and the pellets were washed with 5 ml of 10% trichloroacetic acid and recentrifuged. The pellets were then solubilized in 0.1 N NaOH at 37° for 12 hours. Aliquots were then taken for radioactivity measurements in a Packard Tri-Carb liquid scintillation counter. Protein determinations were made by the method of Lowry et al. (13).

RESULTS

Effect of Tyrosinase on Red Blood Cells—When red cells are incubated in the presence of tyrosinase, lysis of these cells occurs. This effect is observed with both human or rat blood cells. The lytic process, as measured by hemoglobin release, follows an enzyme-mediated type of reaction. Fig. 1 shows a time course plot for the lysis of red blood cells by tyrosinase. Under the conditions used, the reaction is linear for 2 hours and then begins to level off. The extent of reaction varied from one red blood cell preparation to another, but was usually around 20%. In some experiments lysis as high as 40% was observed. Control cells usually showed lysis of 1 to 3%. In Fig. 2, the effect of increasing the concentration of tyrosinase on red cell lysis is seen. Lysis is directly proportional to the amount of enzyme present. The effect of increasing the red blood cell concentration is shown in Fig. 3. With increasing concentrations of red blood cells, there is an increase in lysis. To achieve saturation of the enzyme, it is necessary to use cells 4 times more concentrated than shown in Fig. 3.

Table I shows the effect of cyanide on the lysis of red blood cells by tyrosinase. By subtraction of the appropriate control
Effect of cyanide on lysis of red blood cells by tyrosinase

The incubation mixtures contained 0.5 ml of the stock red cell suspension, 2.0 ml of 0.9% NaCl, and sodium phosphate buffer, 0.008 M. The NaCN concentration was 0.001 M. Enzyme concentration was 525 tyrosine units (52 μg). Samples were incubated at room temperature for 2 hours.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Δ A 413</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>0.17</td>
</tr>
<tr>
<td>Control cells + CN-</td>
<td>0.34</td>
</tr>
<tr>
<td>Tyrosinase-treated cells</td>
<td>1.23</td>
</tr>
<tr>
<td>Tyrosinase-treated cells + CN-</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Effect of tyrosinase on glucose uptake by isolated rat diaphragm and epididymal fat pad

The incubation mixture contained two hemidiaphragms in 3 ml of Krebs-Ringer solution containing 2 mg of glucose and either tyrosinase (2000 units, 200 μg) or buffer. Incubation was carried out at 37°C for 90 min (see "Methods"). Each experiment represents the hemidiaphragms or fat pads from 10 animals. In each experiment, p = 0.10 > p < 0.05.

<table>
<thead>
<tr>
<th>Tissue and animal condition</th>
<th>Glucose uptake</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tyrosinase-treated</td>
</tr>
<tr>
<td>mg/g tissue/90 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm, fasted</td>
<td>1.48 ± 0.14</td>
<td>1.06 ± 0.10</td>
</tr>
<tr>
<td>Diaphragm, fed</td>
<td>0.95 ± 0.14</td>
<td>0.53 ± 0.10</td>
</tr>
<tr>
<td>Diaphragm, fed</td>
<td>0.97 ± 0.07</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Epididymal fat pad, fed</td>
<td>0.12 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

Effect of tyrosinase on glycine-2-¹⁴C uptake by isolated rat diaphragm

The incubation mixture contained two hemidiaphragms, in 3.1 ml of Krebs-Ringer solution containing 2 mg of α-glucose, 0.5 μC of glycine-2-¹⁴C, and either buffer or tyrosinase (4400 units, 440 μg). Incubation was carried out at 37°C for 90 min (see "Methods"). The results of two experiments with fed animals are given. In each experiment, p = 0.10 > p < 0.05.

<table>
<thead>
<tr>
<th>Glycine-2-¹⁴C uptake</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/mg tissue/90 min</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Tyrosinase-treated</td>
</tr>
<tr>
<td>205 ± 125</td>
<td>459 ± 135</td>
</tr>
<tr>
<td>208 ± 92</td>
<td>322 ± 81</td>
</tr>
</tbody>
</table>

Effect of tyrosinase on glycine-2-¹⁴C incorporation into protein of isolated rat diaphragm

The incubation mixtures are the same as in Table III. The results of two experiments are given.

<table>
<thead>
<tr>
<th>Glycine-2-¹⁴C incorporated into protein</th>
<th>Tyrosinase-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/μg</td>
<td>186</td>
</tr>
<tr>
<td>Control</td>
<td>177</td>
</tr>
<tr>
<td>Tyrosinase-treated</td>
<td>193</td>
</tr>
</tbody>
</table>

values, it is seen that lysis of red cells is inhibited 78% by cyanide (which at 0.001 M is a potent inhibitor of tyrosinase) (14).

Red cell lysis by tyrosinase was only partially prevented by the addition of ascorbate (1 × 10⁻⁴ M) to the incubation mixture. The percentage of lysis in the presence of ascorbate and tyrosinase was 70% of that of the lysis observed in the presence of tyrosinase alone. The addition of the tyrosinase-generated quinone of N-acetyl-D-tyrosine (4 × 10⁻⁴ M) to the red cells in the absence of tyrosinase did not cause lysis of the blood cells.

Heat-denatured tyrosinase had no effect on red blood cells.

Effect of Tyrosinase on Glucose Uptake by Isolated Rat Diaphragm—Incubation of isolated rat diaphragm with tyrosinase causes an inhibition of glucose uptake by these membranes. The range of inhibition of tyrosinase-treated diaphragms is from 28 to 44% of the uptake by the control diaphragms. These results are shown in Table II. Also included in Table II is the effect of tyrosinase on glucose uptake by isolated epididymal fat pads. In this case, the percentage inhibition is higher (75%). All differences are significant at <0.001 level.

Effect of Tyrosinase on Glycine-2-¹⁴C Uptake by Isolated Rat Diaphragms—In the presence of tyrosinase, glycine-2-¹⁴C uptake by the rat diaphragm is markedly stimulated. These results are shown in Table III. There is about a 50% increase in glycine uptake by the tyrosinase-treated diaphragms, there is no increase in specific activity of the protein of these diaphragms. These data are shown in Table IV.

Effect of Tyrosinase on Ehrlich Ascites Cells—Incubation of Ehrlich ascites cells with tyrosinase causes a decrease in viability, as measured by eosin dye uptake, with a swelling of the cells.

Discussion

These data provide evidence that highly purified mushroom tyrosinase has a marked effect on mammalian cell membranes. From the results obtained with the red blood cells, it is concluded that red cell lysis in the presence of tyrosinase is the result of an enzymatic reaction. This is based on the facts that (a) there is a linear relationship between lysis and tyrosinase concentration; (b) there is an increase in lysis with the increase in red cell concentration with the eventual saturation of the enzyme; (c) lysis by tyrosinase is inhibited by an inhibitor of tyrosinase (CN⁻); and (d) heat-denatured tyrosinase has no effect on red cells.

The results obtained with the isolated rat diaphragm show that the red cell membrane is not the only membrane system affected by tyrosinase. It is not clear why there is an inhibition of glucose uptake and a stimulation of glycine uptake. However, it has been shown in other types of studies that the glucose and amino acid transport sites were independent of each other. For example, Wool and Moyer have shown that actinomycin D has no effect on glucose uptake by the rat diaphragm but stimulates the transport of α-aminoisobutyric acid into the diaphragm (15). Since glycine uptake in tyrosinase-treated diaphragm membranes was stimulated about 50% (Table III) and there was no increase in the specific activity of the protein isolated from these membranes (Table IV), it is clear that it is only the transport process which is affected and not the other functions of the cell. On the other hand, insulin, which affects the transport processes, also affects the processes of protein synthesis, glycogen synthesis, etc. (16).

The effect of tyrosinase on Ehrlich ascites cells is a third ex-
ample of a membrane system that is affected by tyrosinase. In addition to increasing eosin dye uptake (which in this instance may or may not be an index of cell viability) there is a pronounced swelling of the ascites cells in the presence of tyrosinase.

It has been shown that the tyrosyl residues of a considerable number of intact proteins are oxidized by tyrosinase (3-5). The product of this reaction is the quinone when the tyrosine group is not NH_2-terminal (4).

\[ \text{TYROSINASE} \rightarrow \text{Quinone} \]

In some cases, loss of biological activity of the protein substrate accompanies the oxidation (3, 7). Gross effects, such as red cell agglutination, were earlier reported by Sizer et al. (8). These data show very clearly that tyrosinase has a marked effect on cell membrane permeability or its integrity. Since the specificity of tyrosinase is such that none of the other amino acid side chains of the proteins are affected, these observed effects may be the result of some of the tyrosine residues of the protein layer in the intact membrane being oxidized by tyrosinase.

From electron microscopic studies, it has been established that the cell membrane structure consists of a lipid layer between two protein layers (17). The tyrosyl residues of the peptide and protein components of the membrane would be on the surface and accessible to tyrosinase. It is tentatively concluded that the tyrosyl residues of the cell membrane are involved in cell membrane permeability or its integrity.

However, the possibility has not been excluded that the observed effects are secondary effects in which the oxidized tyrosine groups in the membrane protein react nonenzymatically with some other essential membrane component. The addition of ascorbate at a relatively high concentration (1 x 10^-4 M) could only partially prevent tyrosinase-catalyzed lysis of the red cells. This concentration of ascorbate would be expected to be sufficient to reduce the quinone derivative formed back to the 3,4-dihydroxyphenylalanyl compound in the protein. Another alternative explanation is that the tyrosine residues in the membrane protein are not oxidized at all, but that the free tyrosine is oxidized and the dopa-quinone formed then reacts with other essential components on the membrane. In the red blood cell system, this possibility appears to be ruled out since the addition of exogenous quinone did not cause the lysis of the red cells.

A further complicating factor in the glycine uptake experiments could be the nonenzymatic reaction between the tyrosinase-generated quinone of the tyrosyl residues in the membrane protein or of free tyrosine in the membrane. This nonenzymatic reaction, elucidated by Mason and Peterson (18), would tend to make it appear that there was an increase in glycine uptake. This possibility has not been excluded, but it would not account for the inhibition of glucose uptake which is observed in the same diaphragm system.

Acknowledgments—The author would like to thank Dr. Earl Frieden for his help and support during this investigation. Also, the help of Miss Ann Henderson (now Mrs. J.G.C.) in the early phases of this work is gratefully acknowledged.

REFERENCES
Evidence for a Role of Tyrosyl Residues in Cell Membrane Permeability
Joseph G. Cory


Access the most updated version of this article at http://www.jbc.org/content/242/2/218

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/242/2/218.full.html#ref-list-1