Isolation and Partial Characterization of a Glyceraldehyde 3-Phosphate Dehydrogenase-inactivating Factor from Ehrlich Ascites Tumor Cells

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SUMMARY

A glyceraldehyde 3-phosphate dehydrogenase-inactivating factor has been isolated from the 105,000 X g supernatant solution of Ehrlich ascites tumor cells. This factor was purified 35-fold by steps that include the following: (NH₄)₂SO₄ fractionation; freezing, thawing, and dialysis at 37°; and, finally, filtration chromatography through Sephadex G-100. The factor had a maximum absorption at 280 ml, was not dialyzable, was heat labile, and could be precipitated by 10% trichloroacetic acid. The factor had no detectable proteolytic activity with denatured hemoglobin or a-benzoyl-DL-arginine-p-nitroanilide and was not affected by N-ethylmaleimide or soybean trypsin inhibitor. Excess NAD⁺ was required for complete protection of purified Ehrlich ascites tumor glyceraldehyde 3-phosphate dehydrogenase from inactivation by factor. Glyceraldehyde 3-phosphate dehydrogenase purified from rabbit muscle, Ehrlich ascites cells, mouse muscle, and yeast showed significantly different sensitivities to inactivation by factor.

INTRODUCTION

Green and Dobrjansky (6) have suggested a role for the action of NADase in the regulation of NAD⁺-requiring enzymes. Their data indicated the presence of a factor in the 105,000 X g supernatant solution from Ehrlich ascites tumor cells which, in the simultaneous presence of NADase, inactivated crystalline rabbit muscle G3PDH. Since G3PDH activity has a central role in the metabolic pathways producing ATP in normal and in tumor tissues, it was of interest and importance to isolate this factor and to determine the extent to which it was active in the control of G3PDH in the Ehrlich ascites tumor cells. Portions of this work have been reported elsewhere (10).

MATERIALS AND METHODS

Materials. Enzymes, substrates, and reagents were obtained from the following sources: from Boehringer-Mannheim, Inc., New York, N.Y., crystalline rabbit muscle G3PDH (EC 1.2.1.12), and crystalline rabbit muscle aldolase (EC 4.1.2.7); from the Sigma Chemical Company, St. Louis, Mo., β-NAD⁺, fructose 1,6-diphosphate, Tris, and EDTA.

Methods. The maintenance of the Ehrlich ascites tumor cell line has been described (5). For this study, NADase (EC 3.2.2.5) was purified from Walker 256 carcinosarcoma from rats by the method of Green et al. (7).

Ehrlich ascites cell G3PDH was isolated from a supernatant solution obtained from a homogenate of ascites cells as described by Green and Dobrjansky (6). Mouse muscle G3PDH was extracted from the muscles of the hind legs of 6-week-old HA/ICR albino male mice by the method of Amelunxen and Carr (1). The G3PDH was purified according to the method of Cori et al. (2) with the following modifications. The (NH₄)₂SO₄ fraction precipitating between 65 to 80% saturation and containing the G3PDH activity was redissolved in 0.05 M Tris-HCl buffer, pH 7.4, and 10 mM EDTA (Tris-EDTA buffer), cleared by centrifugation at 105,000 X g for 10 min, and placed on a Sephadex G-100 column (2.5 x 45 cm). The column was eluted with the Tris-EDTA buffer and 3.0-ml fractions were collected. The G3PDH-containing tubes were pooled and concentrated by ultrafiltration. The concentrated material was reprecipitated from 70 to 90% saturated (NH₄)₂SO₄ solution until the specific activity of the enzyme was 35 to 40 units/mg protein. This value was comparable to that of commercially available crystalline rabbit muscle G3PDH.

Enzyme Assays. G3PDH activity was assayed by the method of Wu and Racker (16). A unit of G3PDH activity is defined as the amount of enzyme that reduces 1.0 μmole of NAD⁺/min under the conditions of the assay. NADase activity was assayed by the method of Kaplan (8) as modified by Green and Bodansky (4). A unit of NADase activity is defined as the amount of enzyme that will hydrolyze 1 μmole of NAD⁺/hr under the conditions of the assay.

The activity of the G3PDH-inactivating factor (hereafter referred to as factor) was assayed by determining the amount of G3PDH activity lost after incubation with the factor for 30 min at 37°. One ml of the standard assay mixture contained 7.5 units of Ehrlich ascites G3PDH; 50 mM Tris-HCl buffer, pH 7.4; 10 mM EDTA, and factor. The Ehrlich ascites G3PDH and all other G3PDH preparations were dialyzed against the Tris-EDTA buffer until free of (NH₄)₂SO₄ before use. A unit of factor activity is defined as that amount of factor which causes the loss of 1 unit of ascites cell G3PDH after incubation for 30 min at 37°.

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Table 1
Purification of factor from Ehrlich ascites tumor cells

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total units</th>
<th>Units/mg protein</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 20–65% (NH₄)₂SO₄ fraction</td>
<td>14.5</td>
<td>841</td>
<td>68</td>
<td>0.08</td>
<td>100</td>
</tr>
<tr>
<td>2. Frozen-thawed, dialyzed at 37°</td>
<td>14.5</td>
<td>725</td>
<td>129</td>
<td>0.18</td>
<td>190</td>
</tr>
<tr>
<td>3. Supernatant from Step 2</td>
<td>11.0</td>
<td>220</td>
<td>94</td>
<td>0.42</td>
<td>138</td>
</tr>
<tr>
<td>4. First Sephadex G-100 filtration</td>
<td>60.0</td>
<td>60</td>
<td>102</td>
<td>1.70</td>
<td>150</td>
</tr>
<tr>
<td>5. Second Sephadex G-100 filtration</td>
<td>6.0</td>
<td>36</td>
<td>110</td>
<td>3.00</td>
<td>160</td>
</tr>
</tbody>
</table>

*One unit of factor activity is that amount which causes a decrease of 1 unit of G3PDH under conditions of the assay (see “Materials and Methods”).

RESULTS

Isolation and Purification of Factor. A summary of the results of a typical purification procedure is shown in Table 1. The factor in the 105,000 x g supernatant solution from Ehrlich ascites cells was recovered as the precipitate by (NH₄)₂SO₄ precipitation between 20 and 65% saturation. The precipitate was redissolved in the Tris-EDTA buffer, dialyzed against the same buffer until free of (NH₄)₂SO₄, and centrifuged at 105,000 x g for 10 min (Table 1, Step 1). This material was tested for the presence of NAD-degrading activity (4); none was found. The factor was frozen and thawed 3 times, then dialyzed for 24 hr at 37° against 2 successive 100-volume changes of the Tris-EDTA buffer, and made up with buffer to the original volume (Step 2). A 2-fold increase in total factor activity was obtained by this treatment (Table 1, Step 2, Column 4). This increase suggests an activation of factor. The contents of the bag were centrifuged at 105,000 x g for 30 min to remove the insoluble material (Step 3); some of the factor may have been lost in this material. The factor in this solution was completely precipitated by addition of solid (NH₄)₂SO₄ to 100% saturation, centrifuged, redissolved in a minimal volume of the Tris-EDTA buffer, and dialyzed free of (NH₄)₂SO₄. The solution was then placed on a Sephadex G-100 column (2.5 x 75 cm) and eluted with Tris-EDTA buffer. Fractions of 3 ml were collected and assayed for factor activity. The fractions with activity were pooled (Step 4), concentrated, and rechromatographed through the same column (Step 5). The factor from the last step represented approximately 35-fold purification based on the activity present in the starting material. Again, some activation may have occurred during these latter steps.

Partial Characterization of the Factor. Initial studies with a preparation of the purified factor showed that it had a maximum absorption at 280 nm, was not dialyizable, was inactivated by exposure to 100° for 1 min, and was precipitated by 10% trichloroacetic acid. The elution volume of the purified factor corresponded to a molecular weight of not less than 105,000 when the factor was passed through a standardized Sephadex G-100 column (2.5 x 75 cm) by the method of Whitaker (15). Inactivation of G3PDH increased with the amount of factor used but was not strictly proportional (Chart 1).
The possibility that the factor contained available sulfhydryl groups which were required for its activity was studied. A solution containing 10 mg of purified factor protein in a final volume of 1.0 ml at pH 7.4 was incubated at pH 7.4 for 30 min at 25° with 1.0 mM N-ethylmaleimide. After treatment, excess N-ethylmaleimide was removed by dialysis against the Tris-EDTA buffer, and the ability of the treated factor to inactivate G3PDH was determined. No significant decrease in factor activity was found, suggesting that available sulfhydryl groups were not required for activity.

It was reported previously that trypsin inactivated rabbit muscle G3PDH only when the bound NAD+ was removed from the molecule by charcoal or hydrolyzed by NADase (7). Purified factor was tested to determine whether it acted in a manner similar to trypsin. The factor did not hydrolyze denatured hemoglobin during incubation at 37° for 60 min, either at pH 7.4 or 4.5. In addition, no activity was detectable at pH 7.4 when the chromogenic substrate for trypsin, a-benzoyl-DL-arginine-p-nitroanilide hydrochloride, was used (3), nor was the activity of the factor inhibited by soybean trypsin inhibitor (Table 2). In contrast to our findings that rabbit muscle G3PDH is inactivated by trypsin in the presence of added NADase (7), we observed that the addition of NADase was not necessary for the inactivation of Ehrlich ascites cell G3PDH by trypsin (Table 2, Line 2). Since there was no NAD-degrading activity in this preparation, the possibility existed that G3PDH from different tissues or species may vary greatly in susceptibility to inactivation by proteolytic enzymes.

Inactivation of G3PDH from Different Sources by Ehrlich Ascites Factor. The extent of inactivation by G3PDH's from rabbit muscle, Ehrlich ascites tumor cells, mouse muscle, and yeast by the purified factor is shown in Table 3. Thus, 42% of the Ehrlich ascites G3PDH activity remained after inactivation by the factor alone, whereas the enzyme from yeast was not affected (Table 3, Line 2). Rabbit and mouse muscle G3PDH's showed intermediate degrees of inactivation. The simultaneous presence of added NADase and factor in the incubation mixture (Table 3, Line 4) resulted in additional inactivation of G3PDH from Ehrlich ascites cells, mouse, and rabbit muscle and none in the yeast enzyme. The concentration of NAD+ in G3PDH obtained from rabbit muscle, mouse muscle, and Ehrlich ascites cells was 7.0 to 7.2 μg/mg enzyme protein; none was found in the G3PDH from yeast.

The addition of various amounts of NAD+ in amounts equal to or higher than 6.6 μg to the incubation mixture protected Ehrlich ascites cell G3PDH completely against the inactivation by factor. This added NAD+ was 4 times the amount of NAD+ intrinsically present in the Ehrlich ascites tumor G3PDH. The inactivation of Ehrlich ascites cell G3PDH by factor could be

### Table 2

<table>
<thead>
<tr>
<th>Additions to incubation mixture</th>
<th>G3PDH activity after incubation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin</td>
<td>6</td>
</tr>
<tr>
<td>Trypsin + soybean trypsin inhibitor</td>
<td>92</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>91</td>
</tr>
<tr>
<td>Factor</td>
<td>42</td>
</tr>
<tr>
<td>Factor + soybean trypsin inhibitor</td>
<td>41</td>
</tr>
</tbody>
</table>

* Incubation mixtures contained 7.5 units of Ehrlich ascites cell G3PDH and the following, where indicated: trypsin, 5 μg; soybean trypsin inhibitor, 25 μg; factor, 4 units. Each mixture was made to a final volume of 1.0 ml with 0.15% bovine serum albumin buffered to pH 7.4 with Tris-EDTA.

b Enzyme activities were determined in duplicate before and after 30 min of incubation at 37°. The results are presented as percentage of the control activity after incubation.

### Table 3

<table>
<thead>
<tr>
<th>Additions to incubation mixture</th>
<th>Rabbit muscle</th>
<th>Ehrlich ascites cells</th>
<th>Yeast</th>
<th>Mouse muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Factor</td>
<td>90 ± 2</td>
<td>42 ± 2</td>
<td>97 ± 2</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>NADase</td>
<td>94 ± 3</td>
<td>74 ± 4</td>
<td>99 ± 1</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>Factor + NADase</td>
<td>76 ± 4</td>
<td>18 ± 1</td>
<td>99 ± 1</td>
<td>39 ± 3</td>
</tr>
</tbody>
</table>

* Specific activity of each preparation of G3PDH was between 35 and 40 units/mg protein and contained the following amount of NAD+ in μg/mg enzyme protein, respectively: rabbit muscle, 7.2 ± 0.2; Ehrlich ascites cells, 7.1 ± 0.3; mouse muscle, 7.0 ± 0.4; yeast, none detectable. Each value of NAD+ is the mean ± S.D. of duplicate analysis of at least 2 separate batches of the enzyme preparation.

b Enzyme activities were determined before and after incubation at 37° for 30 min. Activities are expressed as percentage of the control activity after incubation. Each value is the mean ± S.D. of duplicate analysis in at least 2 separate experiments.

c Each incubation mixture contained 7.5 units of G3PDH enzyme activity. Where indicated, 5 units of factor activity and 1.0 unit of NADase activity were added. Each mixture was made up to a final volume of 1.0 ml with 0.15% bovine serum albumin buffered to pH 7.4 with Tris-EDTA.
stopped after 15 min by the addition of NAD*, and the inactivation by the factor resumed when NADase was added to the mixture.

DISCUSSION

Racker and Krimsky (12) reported the presence of a factor in homogenates of brains of mice infected with the Lansing strain of poliomyelitis virus, which inhibited glycolysis when added to homogenates of normal mouse brain. This factor inhibited G3PDH and was not due to inactivation by proteolytic destruction (13). Green and Dobrjansky (6) have reported the presence of a factor in the 105,000 X g supernatant solution from Ehrlich ascites tumor cells which, in the simultaneous presence of NADase, inactivated rabbit muscle G3PDH. This paper describes a method for the partial purification and characterization of this factor. Evidence has been presented which indicates that the factor is a protein but is not trypsin. On the basis of an average molecular weight of the G3PDH as 140,000 (14), and the amount of protein in our enzyme preparation, the concentration of G3PDH as a possible substrate for proteolytic action by the factor cannot be more than 1.4 X 10^-6 M. Hence, very sensitive techniques would be necessary to detect the end products if the factor activity was proteolytic. The finding that the rabbit muscle and Ehrlich ascites cell G3PDH's have the same content of NAD* yet have markedly different sensitivity to inactivation by factor suggested different loci or different modes of binding of NAD*. Additional information is necessary before the mechanism of inactivation of G3PDH by factor is understood.

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REFERENCES

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