Quinoprotein D-glucose dehydrogenase in *Acinetobacter calcoaceticus* LMD 79.41: the membrane-bound enzyme is distinct from the soluble enzyme

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1. SUMMARY

*Acinetobacter calcoaceticus* is known to contain soluble and membrane-bound quinoprotein D-glucose dehydrogenases while other oxidative bacteria such as *Pseudomonas* or *Glucobacter* contain only membrane-bound enzyme. The two different forms were believed to be the same enzyme or interconvertible. Present results show that the two different forms of glucose dehydrogenase are distinct from each other in their enzymatic and immunological properties as well as in their molecular size.

The two enzyme forms were separated after French press-disruption by repeated ultracentrifugation. The soluble and membrane-bound enzymes showed different properties including substrate specificity, kinetics for glucose, and reactivity for ubiquinone-homologues. Importantly, the distinct properties of both enzymes could be retained even after partial purification of the soluble enzyme and solubilization of the membrane-bound enzyme with Triton X-100. Furthermore, the two forms could be distinguished immunologically; the membrane-bound form contains a polypeptide of 83 kDa cross-reactive with antibody raised against *Pseudomonas* enzyme while the soluble enzyme may be a 55 kDa peptide which is not cross-reactive with the antibody.

2. INTRODUCTION

Bacterial D-glucose dehydrogenase (GDH) is a quinoprotein, having pyrroloquinoline quinone as the prosthetic group, and is linked to the respiratory chain. The GDH has been found in a wide variety of bacteria, in which the enzyme is usually tightly bound to the outer surface of the cytoplasmic membrane [1]. Unlike other bacteria, however, *Acinetobacter calcoaceticus* contains a soluble form of GDH in addition to the membrane-bound form [2,3]. The soluble GDH was originally purified by Hauge [2,4] and recently has...
been purified by two groups [5,6]. Thus it has been shown that the soluble enzyme is a dimer of identical subunits, which have a molecular weight of 48,000–54,000, and is able to oxidize disaccharides as well as monosaccharides. On the other hand, attempted purification of the membrane-bound form by solubilization and fractionation, Hauge and Hellberg [7], revealed the enzyme to be the same as the soluble form. Recently, Dokter et al. [8] made a similar observation in which the enzyme in the membrane vesicles turned out to be able to oxidize disaccharides as well as monosaccharides after solubilization with Triton X-100.

However, our recent observation with immunoblotting using antibody prepared against Pseudomonas GDH showed that the membrane of A. calcoaceticus contained GDH peptide of higher molecular weight than the soluble form, which seemed to be closely related to other membrane-bound GDHs [1].

We have, therefore, examined the relationship between the membrane-bound and soluble GDHs in A. calcoaceticus LMD 79.41. The results show that the enzymes are different in terms of molecular size, substrate specificity, kinetics and electron donor specificity.

3. MATERIALS AND METHODS

3.1. Bacterial strain and growth conditions

A. calcoaceticus LMD 79.41 was kindly provided by Dr. J. Duine. The organism was grown at 30°C aerobically to the late exponential phase. The growth medium prepared with tap water included 0.54% sodium succinate, 0.4% ammonium sulfate, 0.14% K₂HPO₄, 0.08% KH₂PO₄ and 0.02% MgSO₄·7H₂O. Usually final klett unit and medium pH were 250–270 and 7.7–7.9, respectively, after 18 h growth.

3.2. Preparation of membrane and soluble fractions

The cells were collected by centrifuging and washed with distilled water, then with 50 mM potassium phosphate, pH 7.5. The washed cells were suspended in 50 mM potassium phosphate, pH 7.5, at a concentration of 1 g wet weight/5 ml. The suspension was passed twice through a French pressure cell press at 16,000 psi, and then centrifuged at 12,000 × g for 20 min to remove intact cells and cell debris. The supernatant was separated into crude membrane and soluble fractions by centrifugation at 12,000 × g for 90 min. Centrifugation was repeated to obtain membrane and soluble fractions from the crude counterparts.

3.3. Preparation of partially-purified soluble enzyme

The soluble fraction was dialysed extensively against 20 mM potassium phosphate, pH 7.2, and then purified by DEAE- and CM-Toyopearl column chromatography essentially as described previously [2,4–6].

3.4. Enzyme assays

GDH activity was measured spectrophotometrically using phenazine methosulfate (PMS) and 2,6-dichlorophenol indophenol (DCIP) as electron acceptors in 50 mM potassium phosphate, pH 6.5, as described in [9]. Quinone reductase activity of GDH was also measured with 50 μM ubiquinone-1 (Q₁) or 20 μM Q₂ in 50 mM potassium phosphate, pH 6.5, as described in [9].

3.5. Other analytical procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in [1]. Immunoblotting analysis was also performed as in [1] except for adding 0.005% SDS to the transfer buffer in order to facilitate transfer of basic proteins [10]. Protein was determined as in [9].

4. RESULTS

4.1. Substrate specificity of the membrane-bound and soluble GDHs

Substrate specificity of the membrane-bound and soluble GDHs of A. calcoaceticus has been examined by Hauge [7,11] and by Duine [3,8]. In
Table 1
Substrate specificity of GDHs in several fractions of *A. calcoaceticus* and partially-purified soluble GDH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Membrane fraction</th>
<th>Solubilized fraction</th>
<th>Soluble fraction</th>
<th>Purified enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>D-Fucose</td>
<td>110</td>
<td>119</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>79</td>
<td>73</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>81</td>
<td>81</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>D-Ribose</td>
<td>46</td>
<td>54</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>12</td>
<td>13</td>
<td>93</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>4</td>
<td>5</td>
<td>72</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

Activity was measured with each sugar (33 mM) as described in MATERIALS AND METHODS, and recorded as % relative to the activity toward d-glucose. Specific activities of each fraction or enzyme for D-glucose are shown in Table 2.

In this study, the experiment was repeated using soluble and membrane fractions prepared from *A. calcoaceticus* LMD 79.41, and further using partially-purified soluble enzyme and supernatant solubilized with 1% Triton X-100 from the membrane fraction (Table 1). As shown, D-fucose, D-xylose, D-galactose and D-ribose as well as D-glucose were good electron donors for GDH of the membrane fraction, while GDH of the soluble fraction showed a low activity for these sugars but not for maltose and lactose compared with D-glucose. Data also indicated that the substrate specificity of the soluble GDH could be retained after its purification. Most importantly, the substrate specificity of the membrane-bound enzyme was also shown to be retained after solubilization from the membrane with 1% Triton X-100.

4.2. Kinetics for D-glucose of the membrane-bound and soluble GDHs

Lineweaver-Burk plots for glucose were obtained with three different samples including the soluble, membrane and solubilized fractions. Under the experimental conditions, as shown in Fig. 1, the soluble fraction exhibited a curve with negative cooperativity as well as substrate inhibition, which was also observed with the partially-purified soluble enzyme (data not shown). On the other hand, the phenomena were not so obvious in the curves obtained with the membrane or solubilized fraction. The $K_m$ value of the membrane-bound or solubilized enzymes was about 2 mM while the soluble enzyme exhibited a $K_m$ of about 30 mM.

4.3. Reactivity for ubiquinone-homologues of the membrane-bound and soluble GDHs

Reactivities for $Q_1$ and $Q_2$ were determined in comparison with PMS reductase activity with the soluble, membrane and solubilized fractions, and also with partially-purified enzyme (Table 2). As shown, Q reductase activity, relative to PMS reductase activity, was considerably higher in the membrane-bound and solubilized enzymes than in the soluble enzyme. Especially, the Q reductase activity had a bias toward $Q_2$ in the membrane-bound or solubilized enzyme.

4.4. Immunoblotting analysis of the membrane-bound and soluble GDHs

Using an antibody raised against GDH of *Pseudomonas fluorescens*, cross-reactivity of GDHs in the soluble and membrane fractions of *A. calcoaceticus* were examined. As shown in Fig. 2, the band cross-reacted with the antibody appear-
Table 2

Q reductase activities of GDHs in several fractions of *A. calcoaceticus* and partially-purified soluble GDH with glucose as substrate

<table>
<thead>
<tr>
<th>Fractions or enzyme</th>
<th>Reductase activity (units/mg)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMS</td>
<td>Q₁</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>11.30</td>
<td>0.43</td>
</tr>
<tr>
<td>Purified soluble GDH</td>
<td>658</td>
<td>17.00</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>4.55</td>
<td>1.07</td>
</tr>
<tr>
<td>Triton supernatant</td>
<td>9.43</td>
<td>1.54</td>
</tr>
</tbody>
</table>

PMS and Q reductase activities were measured as described in MATERIALS AND METHODS, and shown as specific activity and also as relative activity of Q reductase versus PMS reductase.

The soluble GDH of *A. calcoaceticus* has been purified to homogeneity and characterized well by several groups [2,4–6,11]. On the other hand, the membrane-bound form of the enzyme has not been purified and characterized, and thus seemed to be confused with the soluble enzyme. Hauge and Hallberg [7] tried to purify it after solubilization from the membranes with 1.5% deoxycholate; the enzyme was reported to resemble the soluble one as judged by its substrate specificity. Recently, Dokter et al. [8] have reported that solubilization of the membrane vesicles with 0.02% Triton X-100 changes the substrate specificity of membrane-bound GDH and disaccharides can then be oxidized.

We have solubilized and purified membrane-bound GDHs from *P. fluorescens* [9], *Gluconobacter suboxydans* [12] and *Escherichia coli* [13]. These GDHs are all tightly bound to the membrane, and thus solubilized only with relatively high concentrations of both detergent and salt. Furthermore, these membrane-bound GDHs have a similar molecular weight ranging 83–88 kDa and

![Fig. 2. Immunoblotting analysis of several fractions from *A. calcoaceticus* and also partially-purified soluble GDH with an antibody directed for GDH purified from *P. fluorescens*. Each fraction and partially-purified soluble GDH were subjected to SDS-PAGE and immunoblotted.

1, membrane fraction (8.1 µg of protein and 0.03 unit of activity); 2, solubilized fraction (3.2 µg of protein and 0.03 unit of activity); 3, soluble fraction (2.8 µg of protein and 0.03 unit of activity); 4, soluble fraction (10.8 µg of protein and 0.12 unit of activity); 5, soluble fraction (32.4 µg of protein and 0.48 unit of activity); 6, partially-purified soluble GDH (0.063 µg of protein and 0.03 unit of activity); 7, the same enzyme (0.25 µg of protein and 0.12 unit of activity); 8, the same enzyme (1.0 µg of protein and 0.48 unit of activity).
are closely related each other immunologically [1]. These GDHs seem to function by constituting a primary part of the glucose oxidase respiratory chain, which was demonstrated by a reconstitution experiment of the whole system in *E. coli* [14]. Thus, quinoprotein GDH would be generalized as working while bound to the membrane and linking to the respiratory chain. In this respect, GDH of *A. calcoaceticus* has been considered to be exceptional.

However, the results described here indicate that the membrane of *A. calcoaceticus* contains a distinct enzyme from the soluble form and that the membrane-bound form is similar to GDHs of other bacteria with respect to its reactivity with Q, molecular size and immunogenicity. Importantly, unlike the previous papers of two groups [7,8], the characters of the membrane-bound enzyme were retained even after solubilization with 1% Triton X-100. Thus, it seems that *A. calcoaceticus* cells also have a function GDH in the membrane, which is distinct from the enzyme found in the soluble fraction.

The present data also show that the anti-GDH is able to react with the membrane-bound enzyme but not with the soluble enzyme of *A. calcoaceticus*, suggesting that both forms are structurally different from each other. However, the finding does not necessarily mean that the soluble form is not the degradation product or the precursor of the membrane-bound enzyme. Thus, the origin and function of the soluble enzyme remain unclear at this moment.

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REFERENCES