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Crystalline Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus*†

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ABSTRACT: The soluble form of quinoprotein glucose dehydrogenase (EC 1.1.99.17) from *Acinetobacter calcoaceticus* has been purified 2430-fold to electrophoretic homogeneity. The purified enzyme shows a specific activity of 2600 units/mg of protein, and 45% of the starting activity is recovered. In the presence of polyethylene glycol 6000 the purified glucose dehydrogenase crystallizes readily. Glucose dehydrogenase possesses a molecular weight of 110 000 as determined by sedimentation–equilibrium centrifugation. The enzyme is a dimer of identical subunits. The subunit molecular weight was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be 54 000. Each subunit contains one molecule of pyrroloquinoline quinone. Steady-state kinetic measurements with glucose and the one-electron acceptor Wurster's blue indicate that glucose dehydrogenase operates according to a hexa uni ping-pong mechanism. The K_m values were found to be 3.3 mM for glucose and 0.12 mM for Wurster's blue. At higher concentrations both substrates, glucose and Wurster's blue, cause substrate inhibition.

Glucose dehydrogenase from *Acinetobacter calcoaceticus* is an NAD(P)-independent enzyme belonging to the quinoproteins, a novel class of dehydrogenases that possesses a pyrroloquinoline quinone (methoxatin) as the prosthetic group. The enzyme oxidizes glucose and several other aldoses to their corresponding lactones (Hauge, 1960a,b), is associated with the cytoplasma membrane, and transfers the electrons to a cytochrome *b* (Hauge, 1966b). Glucose dehydrogenase from *A. calcoaceticus* can be isolated in a particulate (Hauge, 1966a) and in a soluble form (Hauge, 1966b). The soluble enzyme accepts artificial electron acceptors like 2,6-dichlorophenolindophenol (Hauge, 1960a) or Wurster's blue (Duine et al., 1979) to reoxidize the reduced pyrroloquinoline quinone. Procedures for the purification of quinoprotein glucose dehydrogenase are reported for *Gluconobacter suboxidans* (Ameyama et al., 1981), *Pseudomonas fluorescens* (Matsushita et al., 1980), and *A. calcoaceticus* (Hauge, 1964; Hauge 1966b; Duine et al., 1979).

We present an improved purification procedure for the soluble glucose dehydrogenase from *A. calcoaceticus* resulting for the first time in an homogeneous, crystalline enzyme with an overall yield of 45%.

Materials and Methods

Chemicals. The inorganic chemicals used were of analytical reagent grade. Sodium succinate, glycine, Triton X-100, and DCIP[1] were obtained from Merck (Darmstadt, Germany), and DEAE-Sephacel, CM-Sepharose CL-6B, phenyl-Sepharose CL-4B, and the LMW calibration kit were from Pharmacia (Uppsala, Sweden). Tris and tricine were purchased from

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[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; LMW, low molecular weight; Tris, tris(hydroxymethyl)aminomethane; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PEG, polyethylene glycol; DMSI, dimethyl suberimidate; DNase, deoxyribonuclease; WB, Wurster's blue; PQQ, pyrroloquinoline quinone; SDS, sodium dodecyl sulfate.

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Sigma (München, Germany) and lysozyme, PEG 6000, DMSI, and guanidine hydrochloride, from Serva (Heidelberg, Germany). *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine dihydrochloride was from Fluka (Buchs, Switzerland), Niax Polyol PPG-2025 from Brenntag (Mühlheim/Ruhr, Germany), and Centricon TM 10 from Amicon (Witten, Germany). DNase I from bovine pancreas (grade II) was obtained from Boehringer (Mannheim, Germany). WB was synthesized according to Michaelis and Granick (1943). Hydroxyapatite was prepared as described by Atkinson et al. (1973). Pyrroloquinoline quinone was obtained through the courtesy of Dr. M. Ameyama, Yamaguchi University, Yamaguchi, Japan.

Organism and Growth Conditions. The strain of *A. calcoaceticus* [formerly described as *Bacterium anitratum*, Hauge (1960a)] was provided by Dr. J. Hauge, Veterinary College of Norway, Oslo, Norway. The organism was grown at 22 °C in a 200-L pilot fermenter with an air flow of 10 L/min and a stirring velocity of 180 rpm. The mineral salt medium described by Duine and Frank (1981) was used, supplemented with 0.02 M sodium succinate as the carbon source. The culture was continuously adjusted to pH 8.5 by adding 4 N HCl during the first few hours and by adding 4 N NaOH later. Niax Polyol was added to a concentration of 50 ppm to prevent foaming. The cells were harvested at the end of the exponential growth phase by a Westfalia separator. Usually an optical density of $OD_{400-600} = 1.5$ was reached after about 18 h. The yield of the 200-L batches varied between 700 and 800 g, and the wet cell paste was stored at −20 °C.

Protein Determination. Protein was determined by a dye-binding method according to Sedmak and Grossberg (1977). Protein samples containing Triton X-100 were determined following the method of Mather and Tamplin (1979). Bovine serum albumin was used as a standard.

Enzymatic Test. The activity of glucose dehydrogenase was measured spectrophotometrically by following the reduction of DCIP at 600 nm or of WB at 610 nm. The test solution contained 50 mM potassium phosphate buffer, pH 6, 20 mM glucose, enzyme, and an electron acceptor, either 40 μM DCIP (Hauge, 1960a) or 0.2 mM WB in a total volume of 1 mL. About 0.15 unit of glucose dehydrogenase was used, and all tests were performed at 25 °C.

Initial rates were measured by following the reaction continuously in a Zeiss Model PMQ III spectrophotometer equipped with a recorder. The enzyme was added to the assay mixture lacking substrate, and the reaction was started after 1 min by the addition of glucose.

In steady-state kinetic experiments various concentrations of glucose and WB were used. The measured velocities were proportional to the enzyme concentration at all concentrations of substrate used.

One unit of glucose dehydrogenase is defined as that amount oxidizing 1 μmol of glucose to gluconolactone per minute at 25 °C, thereby reducing 2 μmol of WB under theoretically saturating concentrations of the substrates.

Activity Staining. To detect glucose dehydrogenase activity after electrophoresis, the gels were immersed in a solution containing 50 mM potassium phosphate buffer, pH 6.0, 20 mM glucose, and 20 mM WB. Enzyme activity is visible as a colorless band on a blue background.

Gel Electrophoresis. SDS gel electrophoresis was carried out according to Laemmli (1970), but no stacking gel was used. Slab gels (10%) were used.

Cross-linking of glucose dehydrogenase was performed as described by Davies and Stark (1970). The molecular weights of the subunit and the cross-linked dimeric enzyme were estimated from a calibration curve established with proteins of known molecular weights (LMW calibration kit).

Centrifugation Procedures. Sedimentation experiments were performed in 20 mM potassium phosphate buffer, pH 7.0, at 20 °C in a Beckman-Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. The glucose dehydrogenase preparation contained 0.3 mg/mL.

Boundary–sedimentation studies were performed with 12-mm double-sector charcoal-filled Epon centerpieces. Sedimentation–equilibrium by the meniscus-depletion method was performed in an equilibrium six-channel charcoal-filled Epon centerpiece. Protein distribution was recorded at 280 nm.

All molecular weight calculations used a partial specific volume $\bar{v} = 0.735$. Densities and viscosities are taken from data in the *International Critical Tables.*

Determination of the PQQ Content. The PQQ content of glucose dehydrogenase was determined in a way similar to that described by Hauge (1964). To samples of glucose dehydrogenase in an anaerobic cuvette in 20 mM potassium phosphate buffer, pH 7.0, containing 20 nmol of subunits were added variable amounts of glucose, and the change in absorbance at 337 nm was registered. The respective change in absorbance was set in relation to the maximal possible change at 337 nm provoked by an excess of glucose.

In addition, the PQQ content was determined by comparing the spectra of isolated PQQ and of glucose dehydrogenase in the presence of 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer, pH 6.5. A molar extinction coefficient for PQQ of $\epsilon_{249} = 18400\ M^{-1}\ cm^{-1}$ was used (Dr. M. Ameyama, personal communication).

Crystallization of Glucose Dehydrogenase. Crystallization was achieved by the hanging-drop method according to McPherson (1976). Samples of 10 μL containing 1.9 mg/mL glucose dehydrogenase and 8% (w/v) PEG 6000 in 36 mM Tris/39 mM glycine buffer, pH 9, were vapor equilibrated against 36 mM Tris/39 mM glycine buffer, pH 9, containing 12–36% (w/v) PEG 6000.

Purification of Glucose Dehydrogenase. Step I: Crude Extract. Wet cells (320 g) were suspended in 480 mL of 36 mM Tris/39 mM glycine buffer, pH 9.0. The suspension was stirred at 20 °C and treated with 0.2 g of lysozyme. After 5 min, 1 mg of DNase I was added. After the mixture was stirred for another 5 min, Triton X-100 was added to 1% (v/v). The mixture was transferred to an ice bath and stirred for an additional 1 h. All subsequent steps were carried out at 4 °C. Nonsolubilized material was removed by centrifugation at 4500*g* for 30 min. The pellet was extracted again with 600 mL of 36 mM Tris/39 mM glycine buffer containing 1% Triton X-100. The two extracts were combined, and the resulting solution was regarded as crude extract.

Step II: DEAE-Sephacel Chromatography. To the crude extract was added 440 mL of DEAE-Sephacel equilibrated with 36 mM Tris/39 mM glycine buffer + 1% Triton X-100. The suspension was stirred for 30 min and placed in a column (7.5 cm × 12 cm). Glucose dehydrogenase, which does not bind to DEAE-Sephacel under the conditions used, was washed off with equilibration buffer at a rate of 200 mL/h.

Step III: CM-Sepharose CL-6B Chromatography. The active fractions of the DEAE-Sephacel eluate were pooled and adjusted to pH 6.0 with 2 M acetic acid. The enzyme extract was applied to a column (5 cm × 20 cm) of 400 mL of CM-Sepharose CL-6B equilibrated with 5 mM potassium phosphate buffer, pH 6.0. The column was washed with the same buffer until no Triton X-100 could be detected spectrophotometrically at 280 nm in the eluate. Glucose dehydrogenase

Table I: Purification of Glucose Dehydrogenase from *A. calcoaceticus*

step	vol (mL)	total protein (mg)	total act. (units)	sp act. (units/mg)	purification	recovery (%)
crude extract	1420	17800	19000	1.1	1	100
DEAE-Sephacel chromatography	1850	9150	16100	1.8	1.6	85
CM-Sepharose CL-6B chromatography	158	53	10800	204	191	57
hydroxyapatite chromatography	73	4.15	8930	2150	2009	47
phenyl-Sepharose CL-4B chromatography	14.5	3.3	8570	2600	2430	45

was eluted with a linear gradient of 25 mM potassium phosphate buffer from pH 6.3 to pH 7.8 + 0.225 M NaCl in a total volume of 1200 mL. The flow rate was maintained at 67 mL/h.

Step IV: *Hydroxyapatite Chromatography*. The active fractions eluted from the CM-Sepharose CL-6B column were loaded onto a column (1.5 cm × 20 cm) containing 35 mL of hydroxyapatite. Elution was performed at a flow rate of 30 mL/h with a linear gradient of potassium phosphate buffer, pH 7.0, from 0.15 to 0.4 M in a total volume of 800 mL.

Step V: *Phenyl-Sepharose CL-4B Chromatography*. To the combined active fractions from the hydroxyapatite column was added solid $(NH_4)_2SO_4$ to give a saturation of 30%. This solution was applied to a column (1 cm × 5 cm) containing about 4 mL of phenyl-Sepharose CL-4B. Glucose dehydrogenase was eluted with a linear decreasing gradient of $(NH_4)_2SO_4$ (30–0%) in a total volume of 62 mL of 0.1 M potassium phosphate buffer, pH 7.0. A flow rate of 4.5 mL/h was maintained. The combined active fractions were concentrated with a Centricon TM 10 to about 1 mg/mL and stored at −80 °C.

Results

Purification of Glucose Dehydrogenase. Table I summarizes the results obtained by applying the purification procedure described under Materials and Methods. The most effective step in the purification scheme is chromatography on CM-Sepharose CL-6B. Glucose dehydrogenase was purified 2430-fold with an overall recovery of 45%. The purified enzyme shows a specific activity of 2600 units/mg, from which a turnover number of 2330 s^{-1} with Wurster's blue as electron acceptor is calculated.

Homogeneity of Glucose Dehydrogenase. The homogeneity of the enzyme preparation was tested by analytical polyacrylamide gel electrophoresis. The enzyme migrates as a single component, and no further protein band is visible when up to 10 μg of protein is applied to a single lane. By the activity staining procedure described under Materials and Methods, only one band of glucose dehydrogenase activity is found. The enzymatic activity corresponds with the stained protein band. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate also reveals but a single band of protein (Figure 1A). In boundary–sedimentation experiments in the analytical ultracentrifuge, glucose dehydrogenase moves as a homogeneous protein with a sedimentation coefficient of $s_{20,w}$ = 5.8 S.

Molecular Weight and Subunit Structure. The molecular weight of the enzyme subunit was found to be 54000 by SDS gel electrophoresis (Figure 1A). When glucose dehydrogenase is cross-linked by dimethyl suberimidate, two bands are found by SDS gel electrophoresis. The molecular weight of the faster moving band was found to be 53000, while the slower moving band shows a molecular weight of 102000 (Figure 1B). The intensity of the slower moving protein band increases with increasing concentrations of cross-linker.

The molecular weight of glucose dehydrogenase was also determined by meniscus-depletion sedimentation–equilibrium in the analytical ultracentrifuge and was estimated to be 110000. Glucose dehydrogenase therefore is a dimer of M_r 102000–110000, consisting of two identical subunits of M_r 54000. It was not possible to estimate the molecular weight of glucose dehydrogenase from molecular sieving experiments with Ultrogel AcA 44 or Sephadex G 200. The enzymatic activity was eluted at positions corresponding to molecular weights ranging from 17000 to 70000, depending on the buffers and their concentrations used.

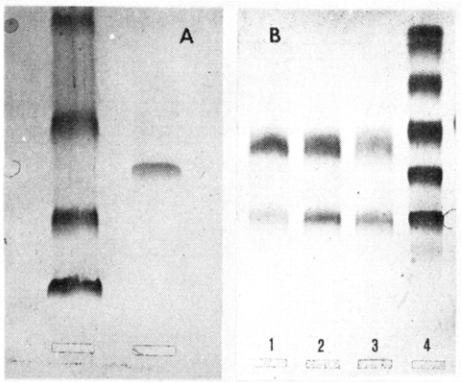


FIGURE 1: SDS gel electrophoresis. (A) Right lane, homogeneous preparation of 10 μg of glucose dehydrogenase. (B) Cross-linked glucose dehydrogenase. Pure glucose dehydrogenase with a concentration of 3 mg/mL was cross-linked with 1, 3, and 6 mg/mL DMSI, treated with SDS, and subjected to SDS gel electrophoresis as shown in lanes 1–3, respectively. A calibration curve for molecular weight determination was obtained with phosphorylase B (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonate dehydratase (M_r 30000), soybean trypsin inhibitor (M_r 20100), and α-lactalbumin (M_r 14400) as shown in lane 4. The electrophoresis was performed from bottom to top.

Determination of the PQQ Content of Glucose Dehydrogenase. Under anaerobic conditions glucose reduces PQQ, the prosthetic group of glucose dehydrogenase. The reduction is accompanied by an absorbance change at 337 nm. The enzyme was completely reduced with an excess of glucose. With increasing amounts of a glucose solution of known concentration, the amount of glucose for complete reduction was calculated. A 19.2-nmol portion of glucose reduces completely 1.08 mg of glucose dehydrogenase. From these data, it is calculated that one subunit of the enzyme contains 0.96 PQQ; therefore, the enzyme contains one prosthetic group per subunit.

The PQQ content of glucose dehydrogenase was also determined by absorption spectroscopy. The absorption spectrum of the enzyme in the presence of 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer, pH 6.5, was compared with the absorption spectrum of PQQ under identical conditions. From the extinction values of both solutions at 350 nm, where the protein does not absorb, the extinction value of PQQ in the enzyme solution was calculated to be 0.119 at 249 nm. From the known extinction coefficient of ϵ_{249} = 18400 M^{-1} cm^{-1}, the concentration of PQQ in the protein solution was calculated to be 6.5 μM. From these data, again a PQQ content of 0.97 per subunit of glucose dehydrogenase was estimated.

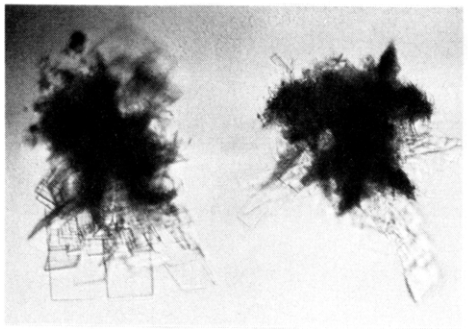


FIGURE 2: Crystals of glucose dehydrogenase from *A. calcoaceticus*. The enzyme was crystallized by vapor equilibration with a solution of 12% PEG 6000 (w/v). 50× magnification.

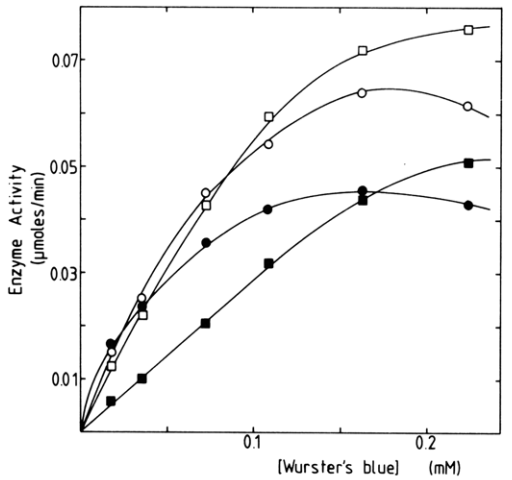


FIGURE 3: Substrate inhibition by WB and glucose. Dependence of the reaction rate on the WB concentration at different concentrations of glucose: 90 (■), 18 (□), 4.5 (○), and 1.8 mM (●).

Crystals of Glucose Dehydrogenase. Crystals of glucose dehydrogenase are obtained by vapor diffusion in hanging drops. Droplets containing 1.9 mg/mL glucose dehydrogenase and 8% PEG 6000 in 36 mM Tris/39 mM glycine buffer, pH 9.0, were incubated over precipitating solutions of 24, 20, 16, and 12% PEG 6000 in 36 mM Tris/39 mM glycine buffer, pH 9.0. Glucose dehydrogenase crystallizes in thin rectangular plates of approximately 0.1 mm × 0.2 mm. The crystals tend to form clusters and so far are too thin to be useful for X-ray diffraction analysis. Work is in progress to obtain crystals suitable for X-ray studies. Figure 2 depicts two typical clusters of thin plates. Redissolved crystals showed no activity. The activity however was restored after removal of PEG 6000. Thereby inactive glucose dehydrogenase was dialyzed against 5 mM potassium phosphate buffer, pH 6.0, and was applied to a CM-Sepharose CL-6B column (1 × 5 cm) equilibrated with the same buffer. Extensive washing led to the removal of PEG 6000. After elution of the protein with 25 mM potassium phosphate buffer, pH 7.8, + 0.225 M NaCl the enzymatic activity was restored.

Kinetic Properties of Glucose Dehydrogenase. Glucose dehydrogenase shows uncomplicated reaction kinetics under the conditions of assay. Within a limited range of enzyme concentration the reaction rate is a linear function of the protein concentration, and no disproportional loss of activity occurs with the dilution of extracts. Glucose as well as the electron acceptors shows substrate inhibition at higher concentrations. Substrate inhibition becomes apparent at concentrations of WB above $2K_m$, while for glucose, concentrations above $3K_m$ are inhibitory. The actual degree of inhibition at a given concentration depends on the concentration of the other substrate (Figure 3).

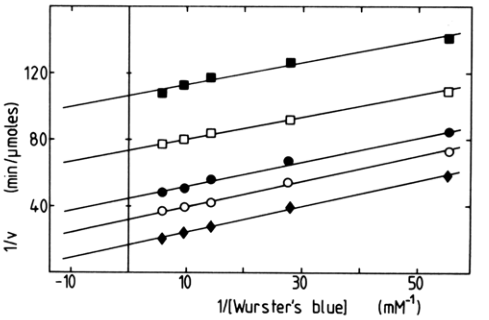
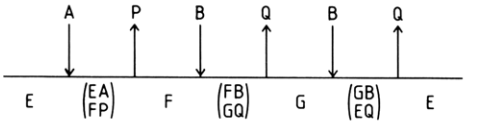


FIGURE 4: Double-reciprocal plot at different concentrations of WB and variable concentrations of glucose: 1.8 (♦), 0.72 (○), 0.45 (●), 0.27 (□), and 0.18 mM (■).

Scheme I: Hexa Uni Ping-Pong Mechanism[a]



[a] Abbreviations: A, glucose; B, WB; P, gluconolactone; Q, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; E, F, and G, stable enzyme forms.

At low concentrations of substrate and electron acceptor, where no substrate inhibition is apparent, the initial velocity follows regular Michaelis–Menten kinetics. There is no evidence of cooperative subunit interaction.

Kinetic Mechanism. Initial rates of the glucose dehydrogenase reaction were determined as a function of the glucose concentration in the presence of various fixed concentrations of WB and vice versa. At relatively low concentrations of glucose and WB, where no substrate inhibition is apparent, the double-reciprocal plots at different concentrations of glucose and variable concentrations of WB and vice versa yield linear parallel lines (Figure 4).

The rate equations for all theoretically possible three substrate mechanisms having two identical substrates are discussed by Görisch (1979) and were derived by the method of Indge and Childs (1976). Only the hexa uni ping-pong mechanism (Scheme I) is compatible with the linear parallel lines in the double-reciprocal plots. Equation 1 gives the rate equation of the hexa uni ping-pong mechanism in the absence of products in a kinetic constant form (A = glucose; B = WB). The kinetic constants were defined as described by Cleland (1963).

$$\frac{V_{max}}{v} = 1 + K_m{}^A\frac{1}{A} + K_m{}^B\frac{1}{B} \quad (1)$$

Kinetic Constants. The kinetic constants defined are determined from linear intercept replots. The K_m value for glucose was found to be 3.3 mM, and the K_m value for WB was found to be 0.12 mM.

Discussion

Quinoprotein glucose dehydrogenase from *A. calcoaceticus* has been purified 2430-fold. Analytical polyacrylamide gel electrophoresis reveals a single protein band. When stained for activity, the activity peak coincides with the protein band stained in a different lane. Analytical gel electrophoresis in sodium dodecyl sulfate also shows one single protein band. Thus, it is concluded that the preparation of glucose dehydrogenase is homogeneous.

The homogeneous preparation of glucose dehydrogenase shows a specific activity of 2600 units/mg with the electron

acceptor Wurster's blue. With DCIP as electron acceptor the preparation shows a specific activity of 4800 units/mg, which is significantly higher than the value of 3400 units/mg reported by Hauge (1964).

The homogeneous enzyme shows a sedimentation coefficient in the analytical ultracentrifuge of $s_{20,w} = 5.8$ S, which is in close agreement with the value of $s_{20,w} = 6.2$ S found by Hauge (1964). Homogeneous glucose dehydrogenase from *A. calcoaceticus* crystallizes readily in the presence of polyethylene glycol.

Our purification procedure of quinoprotein glucose dehydrogenase from *A. calcoaceticus* is based on the results reported by Hauge (1964) and Duine and Frank (1979). To increase the level of the soluble enzyme form of glucose dehydrogenase, *A. calcoaceticus* was grown on succinate at pH 8.5, as described by Duine et al. (1982). $(NH_4)_2SO_4$ precipitation was avoided since this treatment causes large losses of enzymic activity (Hauge, 1964).

After chromatography on DEAE-Sephacel and CM-Sepharose CL-6B Duine et al. (1982) found that the enzyme appeared as one protein on polyacrylamide gel electrophoresis. Despite this apparent homogeneity we found a number of protein bands with this enzyme preparation when polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate. Subsequent chromatography on hydroxyapatite and phenyl-Sepharose CL-4B results in an homogeneous preparation of glucose dehydrogenase, with the highest specific activity reported so far. The molecular weight of intact glucose dehydrogenase was determined by the meniscus-depletion sedimentation–equilibrium method to be 110000. Cross-linking the enzyme with suberimidate followed by SDS gel electrophoresis reveals that the enzyme is a dimer. The molecular weight of the dimer is estimated to be 102000 while a molecular weight of 54000 is found for the subunit.

One pyrroloquinoline quinone molecule was found per subunit of glucose dehydrogenase by titration of the enzyme with glucose. Therefore, the quinoprotein glucose dehydrogenase from *A. calcoaceticus* is a dimer with a molecular weight of about 102000–110000 containing two pyrroloquinoline quinones.

This finding seems to be in conflict with the data reported by Hauge (1964), who determined one prosthetic group per enzyme molecule of M_r 86000. However, the specific activity reported by Hauge (1964) indicates that his preparation contained about 30% of inactive enzyme or of a different protein. In addition, the molecular weight of native glucose dehydrogenase is about 110000 instead of 86000. From these data it is easily shown that quinoprotein glucose dehydrogenase from *A. calcoaceticus* contains two prosthetic groups per native dimer. In addition, our absorption measurements using the known extinction coefficient of the prosthetic group confirm the number of 2 molecules of pyrroloquinoline quinone.

Glucose is oxidized to gluconolactone by the quinoprotein glucose dehydrogenase with the concomitant reduction of a suitable electron acceptor. When DCIP is used as electron acceptor, two electrons are transferred to the redox dye and the enzymatic reaction follows a tetra uni ping-pong mechanism, as was shown by Hauge (1960b). With a one-electron acceptor like Wurster's blue, the oxidation of glucose in a mechanistic sense may be regarded as a reaction with three substrates, Wurster's blue binding twice during the reaction sequence.

This three-substrate reaction, where two substrates are identical, follows an hexa uni ping-pong mechanism. The observed substrate inhibition by glucose and Wurster's blue is in accord with the hexa uni ping-pong mechanism (Cleland, 1970). As reported by Duine and Frank (1980), the methanol dehydrogenase from *Hyphomicrobium* X, another quinoprotein, also operates according to an hexa uni ping-pong mechanism, when one-electron acceptors are involved. Under the test conditions used, glucose dehydrogenase from *A. calcoaceticus* shows about twice the specific activity with DCIP as electron acceptor when compared with Wurster's blue. This behavior may be explained at least partially by the fact that DCIP as electron acceptor is able to reoxidize the reduced prosthetic group by binding once during the reaction sequence, while two molecules of Wurster's blue must bind to accept one electron each.

Quinoprotein glucose dehydrogenase occurs in a variety of microorganisms (Ameyama et al., 1985; Niederpruem & Doudoroff, 1965). In addition to the homogeneous enzyme preparation from *A. calcoaceticus* described in this paper glucose dehydrogenase has been purified to homogeneity or near homogeneity from *P. fluorescens* (Matsushita et al., 1980) and *G. suboxidans* (Ameyama et al., 1981). Both enzymes are hydrophobic proteins, which aggregate in the absence of detergents. In the presence of detergents glucose dehydrogenases from *P. fluorescens* and *G. suboxidans* appear to be monomers with a molecular weight of ~90000. In contrast the purified enzyme from *A. calcoaceticus* is less hydrophobic, since it is soluble in the absence of detergents. This soluble enzyme is a dimer with a molecular weight of 110000; the subunit molecular weight is estimated to 54000. The quinoprotein glucose dehydrogenase from *G. suboxidans* seems to be restricted in its substrate specificity to glucose and maltose (Ameyama, 1981). The enzymes from *P. fluorescens* and *A. calcoaceticus* resemble each other in being aldose dehydrogenases with a rather broad substrate specificity (Matsushita et al., 1980; Hauge, 1960b).

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Cold-Labile Hemolysin Produced by Limited Proteolysis of θ-Toxin from *Clostridium perfringens*†

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ABSTRACT: A nicked toxin whose hemolytic activity is temperature dependent was obtained by limited proteolysis of θ-toxin (M_r 54 000) with subtilisin. The nicked toxin (Cθ) is a complex of two fragments: the N-terminal fragment (M_r 15 000) with basic isoelectric point and the C-terminal fragment (M_r 39 000) with the single cysteinyl residue of the toxin whose reduced form is essential for the hemolytic activity. Cθ hemolyzes erythrocytes only at temperatures above 25 °C, whereas the native toxin hemolyzes them even at 10 °C. At temperatures below 25 °C, Cθ does not hemolyze them although it does bind to membrane cholesterol and although no distinct difference was observed between the secondary structure of Cθ and that of native toxin. It was found that Cθ binds to the cells only in a reversible manner at low temperature, while the native one binds irreversibly to the cells within 10 min, which explains the cold lability of Cθ on hemolysis. The structural basis of the cold lability was discussed through comparison of Cθ with another nicked derivative of θ-toxin that was also obtained.

θ-Toxin (perfringolysin O) is an exotoxin produced by *Clostridium perfringens* type A, which belongs to a group of oxygen-labile or thiol-activated hemolysins (Mitsui et al., 1973; Smyth, 1975; Yamakawa et al., 1977). Other hemolysins that belong to the same group such as streptolysin O (Alouf & Raynaud, 1973), cereolysin (Cowell et al., 1976), tetanolysin (Lucain & Piffaretti, 1977; Mitsui et al., 1980), and alveolysin (Geoffroy & Alouf, 1983) were obtained from culture filtrates of *Streptococcus pyogenes*, *Bacillus cereus*, *Clostridium tetani*, and *Bacillus alvei*, respectively. These hemolysins share common properties (Bernheimer, 1976). They are inhibited by a small amount of cholesterol, activated by thiol compounds, and serologically related to each other (Cowell & Bernheimer, 1977).

In recent years the toxins were reported to damage the cell membrane of human fibroblasts (Thelestam & Möllby, 1980), Hela cells (Duncan & Buckingham, 1980), and myocardial cells (Fisher et al., 1981) in addition to the erythrocyte membrane. However, it is not well understood how they damage the cells after adsorption on the membrane cholesterol. Stimulation of ion efflux (Saito, 1983; Blumental & Habig, 1984) has been reported by several workers, but the existence of a causal relation between these phenomena and cell lysis remains to be established. Since the processes leading to hemolysis after adsorption of the toxin on the membrane cholesterol are sequential and rapid, it is difficult to analyze each process biochemically.

One approach to this problem would be studies with modified toxins that bind but do not hemolyze the cells. They would be useful to distinguish essential processes for hemolysis from secondary effects caused by the toxin action. Another approach is to obtain the toxin fragments that have different biological aspects of the processes. For example, colicins E1, E2, and E3 are found to be composed of three functional domains by analysis of the fragments obtained by the limited proteolysis; each fragment is responsible for each distinctive process of adsorption, penetration, and biological activity in the target cells (Ohno-Iwashita & Imahori, 1980, 1982).

In this study we obtained a nickel derivative of θ-toxin by limited proteolysis and found that it binds but does not hemolyze the cells at low temperatures. The nicked toxin and its constituents were isolated, characterized, and compared with another nicked derivative of θ-toxin that we also obtained.

Experimental Procedures

Materials. Trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] was purchased from Worthington. Soybean trypsin inhibitor, subtilisin Carlsberg, and PMSF[1] were from Sigma. Amopholines were from LKB.

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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