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Proteolytic resistance and its utilization in purification of hydrogenase from *Thiocapsa roseopersicina*

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The effect of various proteinases on the hydrogenase from *Thiocapsa roseopersicina* has been studied by activity measurements accompanied with electrophoretic separation of the protein components. The native enzyme cannot be digested by the following proteinases: thermolysin, subtilisin, trypsin, papain, pepsin, proteinase-K, pronase E, *Bacillus subtilis* proteinase, *Staphylococcus aureus* strain V8 proteinase under standard conditions for optimal proteolytic cleavage. Pretreatments which commonly make resistant proteins accessible to proteolysis have been found ineffective in destabilizing the hydrogenase. The native hydrogenase is readily cleavable and inactivated by CNBr. Also under conditions which irreversibly inactivate the hydrogenase (boiling for 10 min) the enzyme loses its active conformation and becomes susceptible to proteolysis. The protein, as isolated, apparently contains no protective lipids or carbohydrates. The results suggest that a stable hydrogenase apoprotein structure is responsible for the proteolytic resistance. The remarkable stability of this enzyme offers new ways for its purification. A rapid and effective procedure, suitable for scale-up, has been established.

Introduction

Hydrogenases (EC 1.12.-.-) are redox enzymes which catalyze the reversible formation or decomposition of molecular H_2 [1]. A common feature of all hydrogenases studied so far is the presence of at least one iron-sulfur cluster and a polypeptide of similar molecular mass (about 60 kDa). Other structural properties of the functionally related enzymes isolated from various microorganisms are apparently different.

Hydrogenase from the purple sulfur photosynthetic bacterium *Thiocapsa roseopersicina* is a membrane-bound protein [2] in which one 4Fe-4S cluster and one Ni per protein molecule have been found [3]. Unlike many other hydrogenases studied so far, it is a highly heat-resistant and fairly oxygen-stable enzyme [4,5].

Proteolytic digestion is a useful tool to uncover the molecular organization of proteins as well as to prepare peptide fragments for amino-acid sequence analysis and antigenic determinant mapping. Few data are available on the proteolysis of hydrogenases, with the exception of a report that hydrogenase from *Chromatium vinosum* is re-

Abbreviations: CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography.

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sistant to trypsin and pronase [6]. Recently, several hydrogenases have been found to be composed of two subunits [7–10]. The small subunit (about 30 kDa) of the hydrogenases isolated from *Rhizobium japonicum, Azotobacter vinelandii* and *Rhodobacter capsulatus* is susceptible to proteolysis and, if proper precautions are not taken, is lost during the purification procedure. The effect of trypsin on *Escherichia coli* hydrogenase isoenzymes has been thoroughly studied [11,12]. The relevant findings are that trypsin attacks the small (approx. 30 kDa) subunits of both isoenzymes 1 and 2 but does not affect the size of the large (approx. 60 kDa) subunits, nor does the enzyme lose its activity.

Here we report on treatment of native and denatured forms of hydrogenase from *T. roseopersicina* with various proteinases and present evidence that neither the subunit polypeptides nor the enzyme activity of the native enzyme is changed upon proteinase treatment. The application of this remarkable property to devise a new, rapid and efficient purification procedure is described.

Materials and Methods

Strain. T. roseopersicina BBS was obtained from Professor E.N. Kondratieva (Moscow State University, U.S.S.R.). Cells were grown under photosynthetic conditions [13] in 10 liter bottles anaerobically and were collected and stored as in Ref. 14.

Preparation of hydrogenase. The pure hydrogenase for the proteolysis experiments was obtained as described elsewhere [15].

Proteolysis experiments. Stock solutions of proteases (1 mg/ml) in the appropriate buffers were prepared and stored at +4°C. Proteinase activities were periodically checked and stock solutions which digested BSA within 20–30 min at a proteinase-to-BSA ratio of 1:100 (w/w) at 37°C were used in hydrogenase proteolysis experiments.

A list of proteinases used and their respective buffer systems is given in Table I. Incubations were carried out aerobically at 37°C in 1 ml glass vials fitted with silicone rubber discs. Aliquots were withdrawn through the rubber disc at various times and were frozen immediately at −20°C. Samples were analyzed by both activity and either SDS-PAGE or IEF. The proteinase-to-hydrogenase ratio (w/w) was varied in the 1:20 to 1:100 range.

Cyanogen bromide cleavage. 5 mg of CNBr (Fluka, Buchs, Switzerland) was dissolved in 100 μl of 70% formic acid. 100 μg hydrogenase in 400 μl of 70% formic acid was added to it and the reaction mixture was shaken at room temperature for 24 h. Samples were then lyophilized and redissolved in 20 mM potassium phosphate buffer (pH 7.2).

Purification of hydrogenase by trypsin. The crude protein extract [15] was brought to 5% $(NH_4)_2SO_4$ saturation. Phenyl-Sepharose CL4B (Pharmacia, Uppsala, Sweden) suspended in 5% $(NH_4)_2SO_4$ was added to the extract and the suspension mixed at 25°C for 2 h. The gel was separated from the liquid by a glass filter and was packed into a 20 cm K-16 Pharmacia column. The bound proteins were separated with gradient elution using a Pharmacia FPLC as in Ref. 15. The fractions containing hydrogenase were identified by the H_2 evolution assay (see below). The pooled fractions were incubated with lyophilized trypsin (Reanal, Budapest, Hungary) in a 1:20 protein ratio at 37°C for 2 h. The pH of the reaction mixture was adjusted to 7.0–7.5. Solid $(NH_4)_2SO_4$ was added to the proteolysis mixture (final concentration was 5%) and it was then passed through a second Phenyl-Sepharose CL4B column (HR 10/10, Pharmacia). The separations were performed at room temperature, under aerobic conditions.

Hydrogenase activity measurements. An assay system based on H_2 production by a hydrogenase-catalyzed reduction of protons by reduced methyl viologen (Fluka) was routinely used under standardized conditions to avoid systematic errors [14].

SDS-PAGE. The discontinuous buffer system developed by Neville [16] was used: T 14.9, C 1 gels were cast and were stained routinely with Coomassie brilliant blue R-250 (Merck, Darmstadt, F.R.G.) and in some cases with silver [17]. Activity staining by dye reduction was performed according to Ackrell et al. [18]. To detect carbohydrates, the gels were stained with the periodic acid-Schiff's reagent as described in Ref. 24. Prior to electrophoretic separation, proteins were incubated in the presence of SDS and 2-mercaptoethanol (final concentrations 1.0% and

TABLE I

SOURCES OF PROTEINASES AND BUFFER SYSTEMS USED IN PROTEOLYSIS EXPERIMENTS

Residual hydrogenase activities are expressed as percent of similarly incubated, undigested controls. Proteolysis incubation time 1 h, for details see Materials and Methods. Experimental error is ±5%.

Proteinase	Source	Buffer	pH	Residual activity (%)
Thermolysin	Serva	20 mM Tris-HCl + 10 mM $CaCl_2$	8.0	98
Subtilisin	Novo	20 mM Tris-HCl	8.0	101
Papain	Sigma	20 mM Tris-HCl +5 μM cysteine +1 μM EDTA	8.0	100
Trypsin	Merck	20 mM Tris-HCl	8.0	97
Proteinase K	Merck	20 mM Tris-HCl +10 μM EDTA +5 μM 2-mercaptoethanol	7.6	97
B. subtilis proteinase	Calbiochem	0.1 M borate	7.5	99
Pronase E	Merck	0.1 M borate	7.5	98
S. aureus strain V8 proteinase	Sigma	20 mM Tris-HCl	7.5	102
Pepsin	Sigma	0.1 M acetic acid or 0.1 M sodium acetate	6.0	101
Phospholipase a_2	Sigma	10 mM Tris-HCl	7.2	100
Phospholipase *c*	Sigma	20 mM Tris-HCl + 5 mM $CaCl_2$	6.5	100

2.5%, respectively) for 15 min unless otherwise specified.

Isoelectrofocusing in agarose in the pH 3–10 range was performed according to Ref. 19.

Protein concentrations were determined by the method of Hartree [20] using BSA fraction V as a standard (Sigma, Kingston upon Thames, U.K.).

Materials used. The sources of various proteinases are listed in Table I. BSA (fraction V) and phospholipases a_2 and *c*, were purchased from Sigma; chemicals for electrophoresis from Serva (Heidelberg, F.R.G.). Buffer materials were from Calbiochem (La Jolla, CA, U.S.A.).

Results

The following proteolytic enzymes incubated with *T. roseopersicina* hydrogenase for up to 4 h produced no detectable degradation according to SDS-PAGE, IEF and activity measurements: thermolysin, subtilisin, papain, trypsin, proteinase K, pronase E, *B. subtilis* proteinase, and *S. aureus* V8 proteinase.

With each proteinase treatment of the *T. roseopersicina* hydrogenase, BSA was subjected to

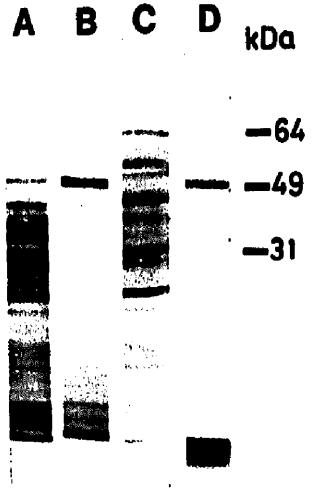

Fig. 1. SDS-PAGE of a heat treated (50 °C, 20 min) BSA/hydrogenase mixture incubated with *S. aureus* V8 proteinase at 37 °C. 80 μg of BSA was mixed with 20 μg of hydrogenase, final volume 100 μl. Incubation times: 10 min (A and C); 240 min (B, D). The proteolytic reaction mixture contained 1% SDS and 2.5% 2-mercaptoethanol in samples A and B. Proteinase-to-hydrogenase ratio was 1:20 (w/w).

proteolysis under identical conditions as controls. Proteolysis parameters were selected so that a complete digestion of the BSA sample was achieved at 37°C within 30 min. In some cases a BSA/hydrogenase mixture was treated with the proteinase to demonstrate that the proteinase retained its full activity in the presence of the hydrogenase preparation. An example of a BSA/hydrogenase mixture digestion is presented in Fig. 1.

The *T. roseopersicina* hydrogenase remained active in the presence of 1% SDS and/or 2.5% 2-mercaptoethanol, i.e., it could be stained for activity following SDS-PAGE provided the samples electrophoresed were not boiled prior to the separation [19]. It was found that hydrogenase was still active after treatment with any of the proteolytic enzymes listed in Table 1 and in fact there was no loss of activity observed when measured by the H_2 evolution assay. Fig. 1 also shows that moderate heat treatment in the presence or absence of SDS and 2-mercaptoethanol before proteolysis did not alter the inaccesibility of the native enzyme (apparent molecular mass, 49 kDa).

Active hydrogenase appears at a position equivalent to 49 kDa on SDS-PAGE, but when it is inactivated by boiling in PAGE buffer [19] (see Discussion) it yields bands at 64 and 34 kDa. The position of any of these hydrogenase bands is unaltered when the enzyme is treated with proteinase for up to 36 h in the active, native state. Furthermore, a careful comparison of the enzyme purified in the presence or absence of proteinase inhibitors (aprotinin, phenylmethylsulfonyl fluoride) showed no noticeable differences. This suggests that there was no digestion of hydrogenase by endogenous proteinases and that we are working with native hydrogenase.

Since there is a 5–10% experimental error of molecular mass determination by SDS-PAGE, it is possible that small peptides, unnecessary for enzymic activity, are cleaved from the polypeptide chain of the native enzyme. This seems unlikely, however, since the sensitive isoelectric point determination shows a p*I* value of 4.15 [19] for all native hydrogenase samples before and after incubation with proteolytic enzymes.

Since hydrogenase shows a remarkable resistance to proteolysis in its native form, the effects of various pretreatments of the enzyme (e.g., heat, SDS, 2-mercaptoethanol, see Fig. 1) were also investigated to see whether proteolysis now occurred.

Upon decreasing the pH to below 4.0, a loss of enzymic activity is observed, probably as a result of destruction of the Fe-S center as detected by loss of absorption at 400 nm and loss of color. Even under these conditions pepsin is unable to digest the native hydrogenase.

A conceivable reason for the unusually high resistance of this enzyme to proteolysis is that lipids or carbohydrates are attached to the protein shielding it from proteolysis. However, treatment of hydrogenase with phospholipase a_2 and/or *c* did not allow proteolysis to occur. To 180 μg of pure hydrogenase was added 10 mU of phospholipase (final volume 100 μl); this mixture was incubated at 37°C for 2 h and then 10 μl of proteinase (1 mg/ml) was added. Incubation with lipases at 37°C for up to 12 h yielded no proteolytic product and the enzyme was fully active. Attempts to detect the presence of carbohydrate in hydrogenase by treatment with known carbohydrate reagents were unsuccessful.

In contrast to its resistance to proteolytic enzymes, the native hydrogenase is easily cleaved by cyanogen bromide (Fig. 2).

Hydrogenase activity is completely and irreversibly lost after boiling the enzyme in air at 100°C for 15–20 min. This pretreatment also eliminates the resistance of our hydrogenase to proteolysis. Boiled hydrogenase is accessible to pronase E, proteinase K (Fig. 2), *S. aureus* V8 proteinase, *B. subtilis* proteinase, papain and trypsin, but not to thermolysin or subtilisin.

The proteolytic resistance of the native hydrogenase has been exploited in a simple and efficient purification scheme for this enzyme (Fig. 3). From 55 g of wet frozen cells the acetone powder (15 g) and a crude protein extract was prepared and subjected to hydrophobic chromatography as described [15]. Some of the strongly bound proteins, including a fraction of the hydrogenase, are not completely eluted from the column by 50 mM Tris-HCl (pH 8.0) (buffer B) and thus a linear gradient of buffer B and H_2O/NaOH (pH 8.0) is required to recover this residual activity. No structural or immunological differences have been observed between the active fractions separated in

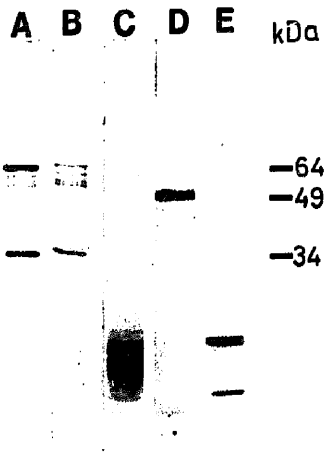

Fig. 2. Cleavage of heat-treated (100 °C, 20 min) hydrogenase by proteinase K (A, B, C) and without heat treatment by CNBr (D, E). Incubation times: 1 min (A), 30 min (B), 300 min (C), none (D), 24 h (E). Proteinase-to-hydrogenase ratio was 1:100 (w/w).

the two peaks in Fig. 3a; therefore, they were combined for subsequent purification steps. The fractions (26 mg protein) were treated with trypsin (1.3 mg) at 37 °C for 2 h with constant agitation. The reaction mixture was applied onto a second Phenyl-Sepharose CL4B (Pharmacia) column in 5% $(NH_4)_2SO_4$ solution. Trypsin and the proteolysed products do not bind to the column and the single major peak eluted contains most of the hydrogenase activity, and electrophoretically 80–90% pure hydrogenase protein can be obtained (4–5 mg).

A homogeneous enzyme is obtained by gel filtration on a Superose 12 HR 10/30 (Pharmacia) column in 50 mM Tris-HCl buffer (pH 8.0)/160 mM NaCl.

The proteinase-purified enzyme shows the same PAGE, IEF, activity, stability and spectroscopic properties as the conventionally purified controls.

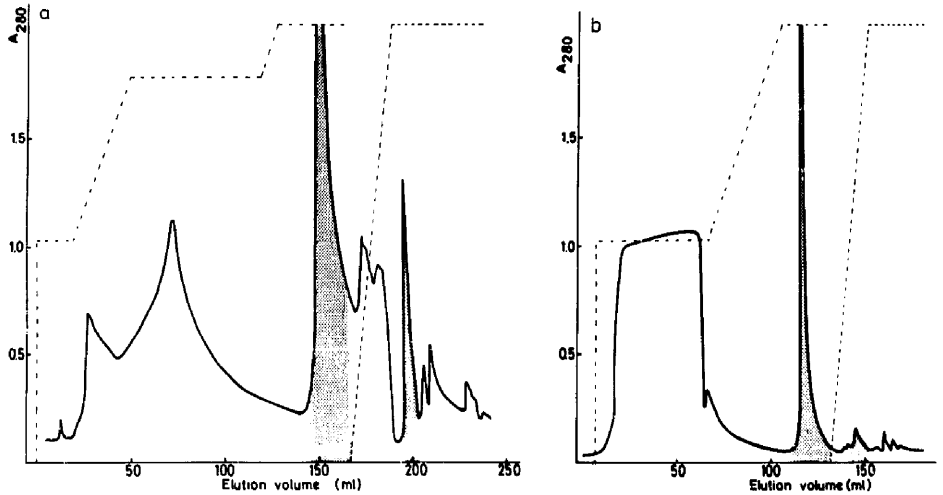

Fig. 3. Hydrophobic interaction chromatography of crude protein extract (a) and trypsin-treated partially purified hydrogenase (b) from *T. roseopersicina*. (a) Crude protein extract was shaken with Phenyl-Sepharose CL4B in 5% $(NH_4)_2SO_4$ for 2 h before the gel was packed into a column (1.6×20 cm). The proteins were separated in a Pharmacia FPLC apparatus aerobically at room temperature. Conditions were: buffer A 5% $(NH_4)_2SO_4$; buffer B 50 mM Tris-HCl (pH 8.0), flow rate 1 ml/min, peak-cutting mode. – – –, gradient profile; ——— protein absorption at 280 nm. The column was subsequently washed with a linear gradient of 50 mM Tris-HCl (pH 8.0) and distilled water/NaOH (pH 8.0). ·–·–·, gradient profile. The dotted area shows H_2 evolving activity. (b) Pooled hydrogenase fractions from the first hydrophobic interaction chromatography were digested with trypsin and the proteolytic mixture was bound to a second Phenyl-Sepharose CL4B column (HR 10/10) in 5% $(NH_4)_2SO_4$. Conditions: Buffer A and B as in (a), flow rate 0.8 ml/min, peak-cutting mode. The gradient profile, protein absorption and hydrogenase activity are indicated as in (a).

Discussion

Hydrogenase from *T. roseopersicina* is remarkably heat-stable [4,5] and has unexpected PAGE behavior [19].

The O_2-stable native enzyme appears at 49 kDa on SDS-PAGE. This is, however, a virtual molecular mass, as upon inactivation (i.e., boiling in SDS-PAGE sample buffer for 10 min) a large and a small subunit are obtained from the 49 kDa protein, having molecular masses of 64 and 34 kDa, respectively [19]. The facts that the large and small subunits carry different antigenic determinants and that they both are parts of the native enzyme has been demonstrated by immunological cross-reactions using antibodies raised against the native enzyme as well as the isolated subunits and by Cleveland peptide mapping (data not shown). These results suggest unusual electrophoretic mobility of the native enzyme. It is intriguing to speculate on what are the structural elements behind these phenomena. Similar behavior of *Azotobacter vinelandii* hydrogenase has been reported recently [21].

The small subunit of hydrogenases of subunit structure similar to ours [7,12] is sensitive to proteinases. The enzyme as isolated from *T. roseopersicina* shows outstanding resistance to proteolytic enzymes with a wide range of origin and specificity. Neither its H_2-evolving activity nor its SDS-PAGE and IEF patterns change under conditions in which BSA is completely decomposed in control experiments. Extending incubation times (up to 100-times that needed for BSA degradation) and applying agents commonly used to destroy the native structure (i.e., moderate heating, 2-mercaptoethanol, SDS) have been equally ineffective both in exposing accessible peptide bonds for proteolytic attack and in activating the hydrogenase function.

Most of the proteinases tested (except for thermolysin and subtilisin) digested hydrogenase after quite severe pretreatment of the enzyme, i.e., boiling the protein solution for 10 min. This pretreatment is accompanied by a complete loss of activity and by unfolding of subunits. Data presented above thus indicate that hydrogenase from *T. roseopersicina* is resistant to proteolysis as long as its activity is preserved. In addition, in certain cases (e.g., low pH, ethanol treatment) the activity is lost while the inactive hydrogenase is still resistant to proteolysis.

There are at least two mechanisms that can explain the proteolytic resistance of hydrogenase from *T. roseopersicina*. One of them is that non-protein components on the enzyme surface protect the peptide bonds. Strongly bound lipids or carbohydrates can play such a protective role. However, attempts to destabilize the hydrogenase by phospholipases and to detect carbohydrates have failed. It is therefore concluded that such a protective mechanism is not substantiated in our case, although the possibility cannot be completely ruled out. The other mechanism involves an unusually stable and compact tertiary structure. It is supported by the preservation of activity under extreme conditions (e.g., heat, SDS) which, together with the unusual electrophoretic mobility of the native enzyme, are indications of a very stable conformation. We assume that strong hydrophobic interactions play a significant role in the stabilization of this protein. Indirect evidence such as in vivo location in the cell membrane, very strong binding to hydrophobic gel matrices, enhancement of activity upon incorporation into liposomes [22] support this view.

Finally, the proteolytic resistance of this hydrogenase can be utilized in the purification of the enzyme which until now was obtained in low yield by conventional chromatographic and electrophoretic techniques. Hydrogenase obtained using a proteinase treatment step is indistinguishable from the enzyme purified in the presence of proteinase inhibitors in all physicochemical properties examined.

The proposed purification procedure is fast and simple, it allows large-scale production of this enzyme, which is a prerequisite to using it in a wide range of biotechnological applications [23].

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