Loss of liposome binding of NADH dehydrogenase from alkalophilic *Bacillus* on subtilisin digestion

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Alkalophile NADH dehydrogenase consisting of two 65-kDa subunits was changed by subtilisin into an enzyme species consisting of two 38-kDa subunits. The amino acid composition and enzyme activity per molecule of the subtilisin-treated enzyme were almost the same as those of the native enzyme, respectively. On mixing with phospholipid liposome, the conformation of the native enzyme was changed, as suggested by the changes in the type of Arrhenius plot and of CD spectrum and enzyme activity. These conformational properties of the subtilisin-treated enzyme, on the other hand, were not affected by liposome. Gel filtration of the subtilisin-treated enzyme mixed with the liposome showed no binding of the protein to liposome.

NADH dehydrogenase *Alkalophilic* Bacillus *Subtilisin digestion* *Liposome binding*

1. INTRODUCTION

Membrane-bound NADH dehydrogenase was purified from alkalophilic *Bacillus* to a homogeneous state [1]. During the purification of the enzyme, it was found that on storage of crude enzyme sample the protein was converted into a smaller enzyme species. The purified enzyme did not exhibit such a size change. The results may indicate proteolytic digestion of the enzyme by proteinase(s) in the bacterium. Here, it was found that the enzyme can be changed into the small enzyme molecule by subtilisin and other proteinases. Some properties, especially on binding to phospholipids, of the small enzyme are presented here.

2. MATERIALS AND METHODS

NADH dehydrogenase was purified from alkalophilic *Bacillus* YN-1 to a homogeneous state, according to [1]. The purified enzyme (2 mg) was treated with 0.4 mg proteinases at 30°C for 1 h in 50 mM phosphate buffer (pH 7.4). Proteinases used were 2-chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), subtilisin BPN′ (EC 3.4.21.14) and Nagase (Nagase, Japan). The proteinase-treated enzyme was subjected to a column chromatography on AMP-Sepharose (2.3 × 7.5 cm). The buffer for equilibration and washing of the column was 50 mM phosphate buffer (pH 7.4) containing 0.1 M KCl and 0.1 mM dithiothreitol (DTT), and the above buffer containing 0.3 mM NAD was used for elution of the enzyme. The enzyme sample thus obtained was subjected to column chromatography on DEAE-cellulose (1 × 3 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.4) containing 0.1 mM DTT and 0.1 M KCl. The enzyme was eluted by 50 mM phosphate buffer (pH 7.4) containing 0.8 M KCl and 0.1 mM DTT.

Membranes were prepared from freshly cultured cells as in [2], and lipids extracted according to [3]. Phospholipids were separated from neutral lipids by acetone precipitation [4]. Native or subtilisin-treated enzyme was mixed with the liposome prepared from membrane phospholipids or dimyristoylphosphatidylcholine (DMPC) in 50 mM phosphate buffer (pH 7.4) containing 0.1 M KCl, as in [5]. When the enzyme mixed with the

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liposome was subjected to gel filtration on Sepharose 4B (1.2 × 14 cm), the liposome was prepared in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 M KCl. Equilibration of the column and elution of the enzyme were carried out with the above Tris buffer.

Assay of enzyme was carried out at 30°C in 20 mM Tris–HCl buffer (pH 8.5) containing 0.15 M KCl. Assay medium contained 250 μM NADH and 40 μM 2,6-dichlorophenolindophenol.

Fluorescence anisotropy (r) of 1,6-diphenyl-1,3,5-hexatriene in the liposome was measured in a Shimadzu RF502 recording spectrofluorimeter, according to [6].

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence and absence of 0.1% SDS at 7.5% gel concentration as in [7] and [8,9], respectively. The electrophoresis calibration kit of Pharmacia was used as protein standard. Activity staining in polyacrylamide gel was performed as in [10].

Cross-linkage of the subtilisin-treated enzyme with glutaraldehyde was carried out according to [11].

The absorption and CD spectra were measured in a Hitachi EPS-3T recording spectrophotometer and in a Jasco J-500C spectropolarimeter, respectively.

Amino acid analysis was carried out as in [1], except for the determination of cysteine and cystine. The amino acid content was determined according to [12], and expressed in half-cystine content.

Flavine, protein and phosphorus were determined according to [1,13,14], respectively.

3. RESULTS AND DISCUSSION

Alkalophile NADH dehydrogenase was treated with trypsin and chymotrypsin, and subjected to AMP-Sepharose column chromatography, and the sample thus obtained was shown to contain a small amount of native enzyme and a large amount of smaller enzyme species, as revealed by PAGE with activity staining. The SDS–PAGE of the sample exhibited two protein bands of 38 kDa and 65 kDa (the subunit of the native enzyme) (fig.1). On treating with Nagase, the native enzyme was shown to split into the 38-kDa component and several smaller components, and the 65-kDa component disappeared almost completely.

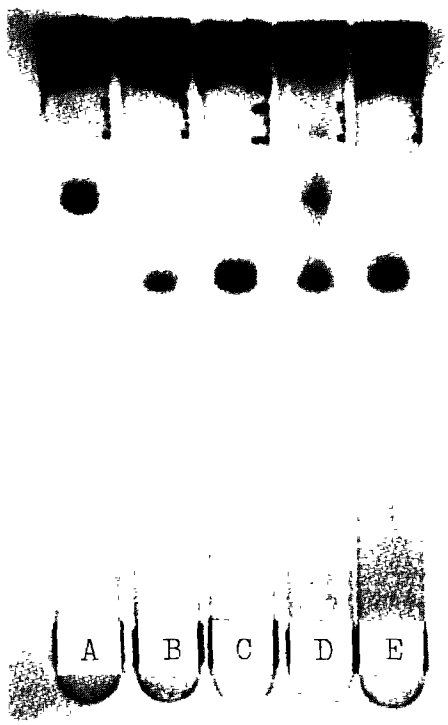

Fig.1. SDS–PAGE profiles of alkalophile NADH dehydrogenase (A) and the enzyme treated with subtilisin (B), chymotrypsin (C), trypsin (D) and Nagase (E). The enzyme samples were charged on the gel at concentrations of 100–200 μg protein.

When the native enzyme was treated with subtilisin, only the 38-kDa component was observed (fig.1). The cross-linkage of the subtilisin-treated enzyme with glutaraldehyde revealed that the small enzyme consisted of two subunits. The results described above indicate that on treating with subtilisin or other proteinases the alkalophile NADH dehydrogenase consisting of two 65-kDa subunits was completely or partially changed into an enzyme molecule consisting of two 38-kDa subunits. Polypeptide(s) of the total size of 27 kDa may have been further digested into smaller peptide fragments.

The 38-kDa enzyme prepared by subtilisin treatment exhibited a specific activity of 130 units/mg protein, and the native 65-kDa enzyme, 75 units/mg protein. Since the molecular size of the native enzyme was 1.8-times larger than that of the

subtilisin-treated enzyme, the activity per molecule of alkalophile NADH dehydrogenase was not changed on subtilisin treatment.

As reported previously [1], the native enzyme contains 1 mol FAD/subunit. The FAD content of the 38-kDa enzyme was determined to be 1.2 mol/subunit. The amino acid composition of the 38-kDa enzyme was essentially similar to that of the native enzyme, and therefore the two enzymes exhibited almost the same polarity (table 1). The native enzyme was activated 22-fold by 150 mM KCl [1]. The 38-kDa enzyme was also activated by 150 mM KCl (15-fold). The results shown above suggest that alkalophile NADH dehydrogenase consists of two subunits, each consisting of two protein domains: the 38-kDa protein domain containing 1 mol FAD and relating to enzyme activity and the 27-kDa domain having no relation to enzyme activity.

Table 1

Amino acid composition of native and subtilisin-treated alkalophile NADH dehydrogenase

Amino acid	Residues per subunit (mol)	
	Native[a]	Subtilisin-treated
Lys	32 (5.2)	22 (6.2)
His	9 (1.1)	6 (1.7)
Arg	18 (2.9)	13 (3.6)
Asx	70 (11.3)	39 (10.9)
Thr	28 (4.5)	19 (5.3)
Ser	45 (7.3)	19 (5.3)
Glx	71 (11.5)	37 (10.4)
Pro	19 (3.1)	11 (3.1)
Gly	74 (12.0)	42 (11.8)
Ala	58 (9.4)	35 (9.8)
Half-cys	3 (0.5)	2 (0.6)
Val	53 (8.6)	32 (9.0)
Met	12 (1.9)	3 (0.8)
Ilu	36 (5.8)	26 (7.3)
Leu	52 (8.4)	27 (7.5)
Tyr	12 (1.9)	8 (2.2)
Phe	19 (3.1)	12 (3.4)
Trp	6 (1.0)	4 (1.1)
Total	617	357
Polarity[b]	44.6	44.3

[a] The amino acid composition for the native enzyme was wrongly calculated in [1]. The corrected values are presented here

[b] Calculated according to [15]

Values in parentheses represent the % compositions

The partially purified NADH dehydrogenase from *B. caldotenax* exhibited a continuous Arrhenius plot, but the enzyme bound to phospholipids displayed discontinuities in the Arrhenius plot [6,10]. The Arrhenius plot of the purified alkalophile enzyme was linear, but the enzyme mixed with the liposome from membrane phospholipids or DMPC exhibited Arrhenius discontinuities at 8 and 31°C or 23°C, respectively (fig.2). The membrane phospholipids exhibited phase transitions at 8 and 30°C (fig.2) and the melting point of DMPC is 23°C [16]. The results shown in fig.2 therefore indicate that the Arrhenius discontinuities of alkalophile NADH dehydrogenase mixed with the liposome are related

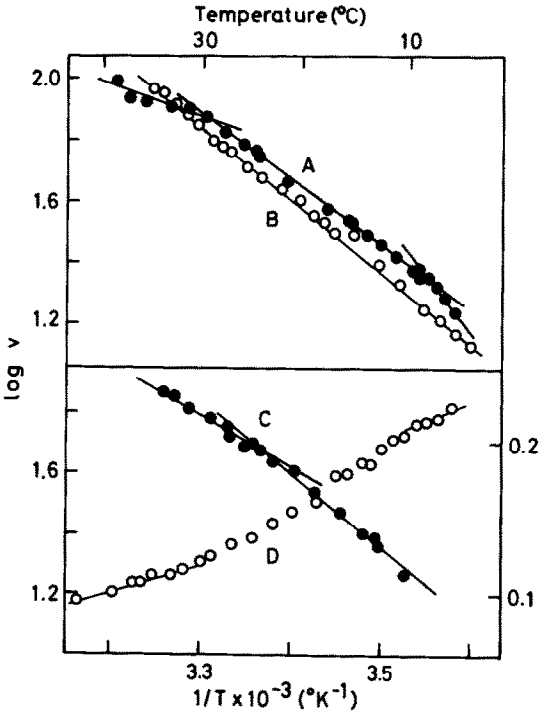

Fig.2. Arrhenius plots for alkalophile NADH dehydrogenase (A) and the enzyme mixed with liposome from membrane phospholipids (B) or DMPA (C), and fluorescence anisotropy (*r*) vs temperature plot for the membrane phospholipids.

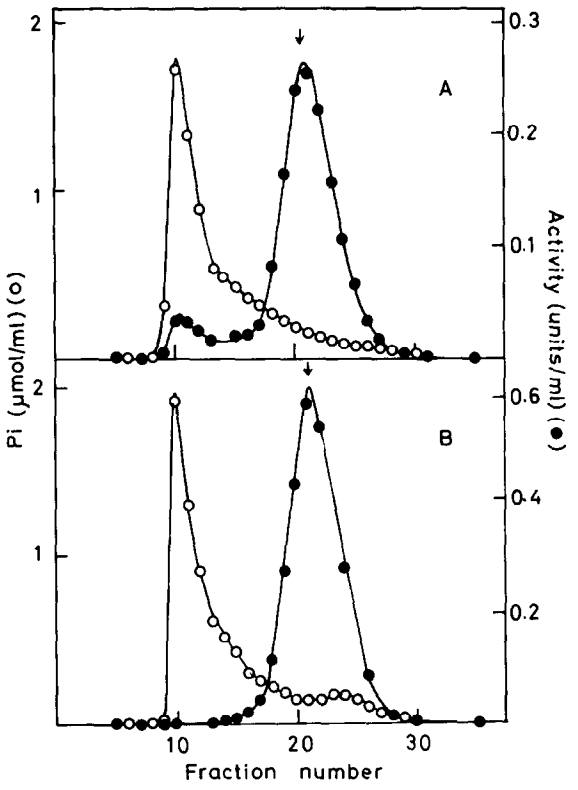

Fig.3. Gel filtration on Sepharose 4B of the native (A) and subtilisin-treated (B) alkalophile NADH dehydrogenases mixed with liposome from membrane phospholipids. 50 μg protein mixed with the liposome from 10 mg membrane phospholipids was used, and each 1 ml fraction was collected. The arrows indicate the elution peaks of the free (A) and free subtilisin-treated (B) enzymes.

to the fluidity change of the liposome with temperature. The 38-kDa enzyme exhibited a continuous Arrhenius plot in the temperature range from 38 to 5°C, with the same slope (activation energy) as that for the native enzyme. In contrast to the native enzyme, the 38-kDa enzyme mixed with the liposome from membrane phospholipids or DMPC exhibited a continuous plot.

The contents of α-helix, β-sheet and remainder estimated from the CD spectrum by the method in [17] for the 38-kDa enzyme were 12, 38 and 50%, and those for the native enzyme 20, 34 and 46% [1], respectively. This indicates a slight conformational change of alkalophile NADH dehydrogenase on subtilisin treatment. When the native enzyme was mixed with the liposome, the CD spectrum of the protein was slightly, but invariably changed [1], indicating a conformational change of the enzyme on binding to liposome. When the 38-kDa enzyme was mixed with the liposome, on the other hand, no change of the CD spectrum was observed. The native enzyme was activated by membrane phospholipids [1]. The 38-kDa enzyme, on the other hand, was not activated by the phospholipids.

When the native enzyme mixed with the liposome from membrane phospholipids was filtered on Sepharose, two enzyme peaks, one with the void volume and another with the elution volume of the free enzyme, were observed (fig.3). The phosphorus peak coincided with the enzyme peak of void volume. A similar gel filtration profile was reported with chemically modified chymotrypsin bound to phospholipid liposome [18]. The results shown in fig.3 indicate that the native alkalophile NADH dehydrogenase was bound to the liposome. When the 38-kDa enzyme was mixed with the liposome and subjected to gel filtration, the enzyme activity was observed only with the elution peak of the free 38-kDa enzyme, and no activity was observed with the liposome fraction (fig.3). As described already, the activity, CD spectrum and continuous Arrhenius plot of the 38-kDa enzyme were not changed by the phospholipids. These results and the result in fig.3 may indicate that the 38-kDa enzyme is unable to bind to the liposome. The polypeptide fragment(s) which may have been digested by subtilisin into much smaller fragments may be related to the property of the alkalophile NADH dehydrogenase binding to phospholipids.

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