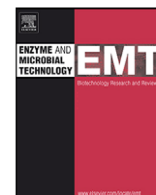
Enzyme and Microbial Technology 46 (2010) 588–593

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt



Stabilization of formate dehydrogenase from *Candida boidinii* through liposome-assisted complexation with cofactors

Makoto Yoshimoto*, Ryo Yamasaki, Manami Nakao, Takayuki Yamashita

Department of Applied Molecular Bioscience, Yamaguchi University, 2-16-1 Tokiwadai, Ube 755-8611, Japan

ARTICLE INFO

Article history:
Received 15 December 2009
Received in revised form 10 February 2010
Accepted 24 February 2010

Keywords:
Formate dehydrogenase
Liposome encapsulation
Enzyme thermal stability
Cofactor
Lipid membrane

ABSTRACT

The activity of formate dehydrogenase from *Candida boidinii* (CbFDH) was stabilized at 60 °C through interaction with its cofactor (NAD^+ or NADH) in the liposomal aqueous phase. The activity of 8.0 μM free CbFDH without liposomal encapsulation progressively decreased at 60 °C in Tris buffer of pH 8.5 following the first-order kinetics. Free CbFDH without cofactor showed the half-life of enzyme activity $t_{1/2}$ of 3.5 min, while $t_{1/2}$ increased to 22 and 236 min with 15 mM NAD^+ and 4.5 mM NADH, respectively. Turbidity measurements revealed that the free CbFDH and CbFDH/NAD^+ became their aggregate-prone states at 60 °C. For the liposomal CbFDH/cofactor systems, the cofactor-induced stabilization of CbFDH was also observed. Typically, the liposomal 6.0 μM CbFDH/4.3 mM NAD^+ showed significantly large $t_{1/2}$ of 36 min compared to the corresponding free CbFDH/NAD^+ ($t_{1/2}$ = 8.9 min). Mixing of free CbFDH/NAD^+ with the enzyme-free liposomes resulted in the insufficient interaction between liposomes and CbFDH showing $t_{1/2}$ of 14 min. The results obtained demonstrate that the lipid membrane assists the formation of highly thermostable enzyme–cofactor complex through stabilizing the structure of the liposome-encapsulated CbFDH.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Formate dehydrogenase (FDH, EC 1.2.1.2) catalyzes the oxidation of formate giving carbon dioxide in the presence of oxidized cofactor NAD^+. The reverse reaction, i.e., the reduction of carbon dioxide is also catalyzed by FDH with an excess of reduced cofactor NADH. The enzyme has considerable potential for biotechnological applications including the regeneration of NADH during enzymatic syntheses of chiral compounds [1], the biological sensing of formate [2] and the production of methanol from carbon dioxide [3–5]. On the other hand, FDH possesses peculiar properties mainly derived from its oligomeric structure. For instance the thermal stability [6,7] and refolding efficiency [8] of FDH from *Candida boidinii* were reported to definitely depend on its concentration which controls the equilibrium between the subunits and dimeric form of the enzyme. These concentration-dependent features of FDH potentially cause difficulties in applying this enzyme as a catalyst in practical bioreactors. FDH and other dehydrogenases were immobilized in various solid supports through covalent coupling [6,7], specific interaction [9] and adsorption [10]. The immobilized dehydrogenases were biologically active, structurally stable and reusable. Cofactor molecules were also covalently immobilized for the enzyme capture [11] and the repeated enzymatic reduction of carbon dioxide [4]. The possible concern of the immobilized enzyme systems is that the enzyme–cofactor interaction is inhibited by the mass transfer resistance and conformational change of the enzyme in solid phase.

Liposomal system possesses an aqueous droplet which is isolated from the bulk liquid with semi-permeable phospholipid bilayer membranes. Enzyme and cofactor molecules can be confined in liposomes in their biologically active forms without employing any chemical modification [12,13]. The liquid volume and the enzyme concentration in liposome can be regulated on the basis of the preparation conditions of the liposomal enzymes. The enzyme concentration in liposome is kept constant regardless of the concentration of liposome if negligible leakage of the enzyme molecules occurs from liposome interior. The dissociation-induced deactivation of FDH [6,7] is thus potentially controlled in the liposomal system on the basis of the liposomal enzyme concentration. These characteristics of the liposomal system imply that it offers a mild environment for the cofactor-dependent oligomeric enzymes.

In the present work, firstly the role of cofactor (NAD^+ or NADH) on the thermal stability of FDH was examined at various cofactor concentrations. The enzyme employed was formate dehydrogenase from *C. boidinii* (CbFDH) that was characterized in its molecular structure and catalytic properties in the previous literatures [14–16]. Secondly, the liposome containing both CbFDH and cofactor was prepared and the thermal stability of the encapsulated enzyme was examined. Then the effect of liposomal encapsulation

* Corresponding author. Tel.: +81 836 85 9271; fax: +81 836 85 9201.
E-mail address: yosimoto@yamaguchi-u.ac.jp (M. Yoshimoto).

0141-0229/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2010.02.012

was clarified on the CbFDH–cofactor complexation at high temperature.

2. Materials and methods

2.1. Materials

Formate dehydrogenase from *C. boidinii* (CbFDH, EC 1.2.1.2, M_r = 74,000, pI = 5.4 [1,16]) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). NAD^+ (β-oxidized nicotinamide adenine dinucleotide) and NADH (β-reduced nicotinamide adenine dinucleotide) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was obtained from NOF Corp. (Tokyo, Japan). Sepharose 4B was obtained from GE Healthcare UK Ltd. (Buckinghamshire, England). All chemicals were used as received.

2.2. Preparation of CbFDH or CbFDH/cofactor-containing liposomes

Fifty milligrams of POPC was solubilized in 4.0 mL of chloroform in a round-bottom flask and the solvent was removed at 35 °C using a rotary evaporator. The POPC was solubilized in 4.0 mL of diethylether and the solvent was removed. This procedure was performed twice. The residual solvent molecules in the POPC film were removed with a freeze-dryer at <8 Pa for 2 h. Then the dry POPC film obtained was hydrated with the 2.0 mL of 50 mM Tris–HCl buffer solution of pH 8.5 (denoted as Tris buffer) containing CbFDH, CbFDH/NAD^+ or CbFDH/NADH. The enzyme and cofactor concentrations were 1.3 mg/mL and 10 mM, respectively. The formation of multilamellar vesicles (MLVs) by hydrating the lipid film was enhanced by freezing at −80 °C for 5 min in dry ice/ethanol and thawing and incubating at 35 °C for 5 min in a water bath (7 cycles). The MLVs were extruded through a polycarbonate membrane with mean pore diameter of 100 nm using an extrusion device Liposofast from Avestin Inc. [17]. The extruded enzyme–liposome mixture was passed through the gel permeation chromatography (GPC) with a sepharose 4B column (1.0 (id) × 20 cm) to remove free enzyme and cofactor molecules. The CbFDH/cofactor-containing liposome suspension (1.0 mL) was loaded on the GPC column and eluted at 25 °C with the Tris buffer solution collecting at 1.0-mL fraction volumes. The flow rate of the eluent was 220 μL/min. The concentration of POPC in each fraction collected was enzymatically determined [18]. Elution of CbFDH and cofactors was detected by measuring the enzyme activity and the absorbance at 260 or 340 nm, respectively (see below). The enzyme-free POPC liposomes were also prepared as described above except that the Tris buffer free of enzyme and cofactor was used in the hydration of a dry lipid film.

2.3. Measurement of cofactor concentration

The concentrations of NAD^+ and NADH, which were encapsulated in liposomes, were determined using a spectrophotometer (V-550, JASCO, Japan) after complete solubilization of the liposome membranes with 40 mM sodium cholate. NADH concentration in the NAD^+/NADH mixture was determined on the basis of A_{340} with the molar extinction coefficient ε_{340} of 6.3×10^3 M^{-1} cm^{-1}. NAD^+ concentration in the mixture was then determined by measuring A_{260} with ε_{260} of 18×10^3 M^{-1} cm^{-1}, taking into account the A_{340}/A_{260} ratio for NADH of 0.43.

2.4. Measurement of protein concentration

Protein content in the commercially available CbFDH was determined according to the method of Bradford [19] using the kit from Bio-Rad Laboratories, Inc. (Hercules, CA). Bovine serum albumin solutions were used as calibration standards. The commercially available CbFDH powder was 13 wt% in the CbFDH content.

2.5. Measurements of liposomal and free CbFDH activities

The CbFDH activities in the liposomal and free enzyme systems were determined based on the CbFDH-catalyzed oxidation of formic acid with NAD^+ producing carbon dioxide and NADH. The assay mixture initially contained 300 mM sodium formate, 1.5 mM NAD^+, 40 mM sodium cholate and the CbFDH sample in the Tris buffer. The sodium cholate induced complete solubilization of the liposome membrane to release the liposomal enzymes. The enzyme reaction was initiated by the addition of CbFDH-containing sample to give 1.5 mL of the above reaction mixture. Then the increase in absorbance at 340 nm was followed for 60 s as an indication of the formation of NADH in a quartz cuvette of 0.5-cm optical length with the spectrophotometer. The temperature of the reaction mixture was maintained at 25 ± 0.3 °C by a perche-type temperature controller (EHC-477T, JASCO, Japan). The initial rate of the NADH formation was taken as the activity of CbFDH. The activity of free CbFDH was determined at various reaction temperatures up to 65 ± 0.3 °C. In this case, to initiate the reaction a CbFDH-containing sample (18 μM, 30 μL) was added to the 2970 μL of the reaction mixture preincubated for 10 min at each temperature using a cuvette equipped with a cap to avoid the evaporation of water. All measurements were performed in triplicate. One unit of the enzyme is defined as the amount of enzyme that converts 1.0 μmol of formic acid per minute at 25 °C. The specific activity based on the commercially available CbFDH powder was 0.30 U/mg. Accordingly, the specific activity of CbFDH was 2.3 U/mg considering the protein content in the commercially available CbFDH powder of 13 wt% (see above).

2.6. Measurements and analysis of thermal stabilities of liposomal and free CbFDH

The Tris buffer solution containing liposomal CbFDH or CbFDH/cofactor at the POPC concentration of 11 mM was incubated in a capped plastic tube placed in a water bath thermostatted at 60 ± 0.3 °C. The initial volume of each enzyme-containing sample was 1.0 mL. The aliquots of the samples were periodically withdrawn and the enzyme activity was immediately measured at 25 °C as described above. The effect of cofactor originally contained in the CbFDH samples on the enzyme activity measurement could be negligible because the cofactor concentration in the sample was significantly diluted in the assay mixture. The thermal stabilities of free CbFDH and CbFDH/cofactor systems were also measured under the identical condition to the above at the enzyme and cofactor concentrations of 3.8–8.0 μM and 0.05–15 mM, respectively. The remaining activity of CbFDH was determined by comparing its activity after the heat treatment with that at 25 °C. The deactivation of CbFDH was analyzed assuming the first-order kinetics, $d[E_t]/dt = -k_d[E_t]$, where $[E_t]$ and k_d stand for the active enzyme concentration at any incubation time t and the deactivation rate constant, respectively. Separation and integration of the above differential equation gives $-\ln([E_t]/[E_0]) = k_d t$, where $[E_0]$ represents the initial concentration of active enzyme. The half-life of the enzyme activity $t_{1/2}$ can be defined as the time needed for the concentration of active enzyme to drop to one-half of the original value and determined as $t_{1/2} = \ln 2/k_d$.

2.7. Turbidity measurement

Turbidity of the Tris buffer solution containing free CbFDH or CbFDH/cofactor was measured at 60 °C based on the optical density at 600 nm (OD_{600}) with the spectrophotometer in order to evaluate the aggregate formation of the enzyme molecules. For all of the turbidity measurements, the sample volume was 3.0 mL, and the capped quartz cuvette with optical path length of 1.0 cm was used.

2.8. Reactivation of thermally deactivated liposomal and free CbFDH

The Tris buffer solution suspending the liposomal CbFDH ([POPC] = 11 mM) was incubated in the capped plastic tube at 60 °C for the time corresponding to $t_{1/2}$. Then the CbFDH-containing liposome suspension was incubated at 20 °C for 20 h to induce the reactivation of CbFDH. The enzyme activity was measured before and after the heat treatment, and after the reactivation period. All activity measurements were performed at 25 °C as described above. The deactivation–reactivation behaviors of free CbFDH were also examined for comparison. It should be noted that the free CbFDH concentration was identical to the enzyme concentration in liposomes.

3. Results and discussion

3.1. Effect of temperature on activity of free CbFDH

Fig. 1 shows the effect of temperature on the activity of free CbFDH at the fixed enzyme concentration of 0.18 μM. In the figure, the maximum activity obtained at 45 °C is taken as 100%. The enzyme activity is seen to be significantly dependent on the reaction temperature. The optimal temperature of the free CbFDH activity is 45 °C at which the activity is 1.9 times larger than that obtained at 25 °C. At 60 and 65 °C, each enzyme activity is significantly smaller than that at 45 °C. This indicates that considerable conformational change in the free CbFDH molecules occurs at the high temperatures.

3.2. Effects of cofactors on thermal stability of free CbFDH

3.2.1. Effect of NAD^+

Effect of NAD^+ on the stability of free CbFDH at the concentration of 8.0 μM was examined at 60 °C at various NAD^+ concentrations up to 15 mM. The time courses of the remaining CbFDH activity are shown in Fig. 2. The broken curves in the figure show the deactivation time courses calculated assuming the first-order kinetics. The figure demonstrates that the thermal deactivation behaviors of CbFDH with and without NAD^+ are apparently approximated by the first-order kinetics. Furthermore, the thermal stability of the enzyme clearly increases with increasing the concentration of NAD^+.

Download English Version:

https://daneshyari.com/en/article/17358

Download Persian Version:

https://daneshyari.com/article/17358

Daneshyari.com