NADH oxidase of *Thermus thermophilus* HB8 overproduced from *Escherichia coli*

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Received March 23, 1994
Accepted July 13, 1994

Summary. An NADH oxidase purified from the extreme thermophile *Thermus thermophilus* HB8 is a monomeric flavoprotein with a 1:1 ratio of flavin-adenine dinucleotide (FAD) to the polypeptide chain. It catalyzes in vitro the oxidation of reduced NADH or NADPH with the formation of H₂O₂. The gene encoding the NADH oxidase from *T. thermophilus* HB8 was cloned, and its nucleotide sequence was determined. The molecular mass of 22,749 Da, as deduced from the *nox* gene, agrees with that of the purified NADH oxidase from *T. thermophilus* HB8, as estimated by mass spectrometry. The *nox* gene does not contain a GX₄GK consensus sequence typical for nucleotide binding proteins. The *nox* gene was overexpressed in *Escherichia coli*, and a protocol for the rapid purification of the *E. coli*-borne *T. thermophilus* NADH oxidase or its His₆-tagged analogue was developed by using thermal denaturation step and affinity chromatography.

Keywords: NADH oxidase; Thermostable flavoprotein; Affinity chromatography; Codon usage.

Introduction

Several bacterial NADH oxidases have been isolated and characterized to date. The NADH oxidase derived from aerobically grown *Streptococcus faecalis* catalyzes the direct four-electron reduction of O₂ to 2 H₂O (Hoskins et al. 1962; Schmidt et al. 1986; Ahmed and Claiborne 1989 a, b). Dolin (1953, 1955) has described another NADH oxidase isolated from anaerobically grown *S. faecalis*. This enzyme catalyzes the reduction of oxygen to hydrogen peroxide. Two other NADH oxidases, isolated from *Bacillus megaterium* (Saeki et al. 1985) and from the thermophile *Thermus aquaticus* YT-1 (Cocco et al. 1988), have been reported to produce hydrogen peroxide with the consumption of oxygen via a two-electron reduction.

The purification, some properties, identification of a gene, and overexpression of the NADH oxidase from *Thermus thermophilus* HB8 in *E. coli* were reported (Park et al. 1992 a, b). Here the corrected gene sequence and the preparation of NADH oxidase carrying a His₆-tag, allowing its rapid purification on Ni²⁺-nitrilotriacetate (NTA) columns are presented.

Material and methods

β-NADH and FAD were obtained from Serva (Heidelberg, Federal Republic of Germany), β-NAD⁺ was purchased from Biomol (Hamburg, Federal Republic of Germany). Q Sepharose Fast Flow, Blue Sepharose CL-6B and Sephacryl S-200 HR were obtained from Pharmacia (Freiburg, Federal Republic of Germany). Fractogel EMD SO₃⁻-650 (M) and lysozyme from hen egg with (100 kU/mg) were from Merck (Darmstadt, Federal Republic of Germany). All other chemicals were of the best grade available from Merck.

Purification of NADH oxidase from *T. thermophilus* HB8 cell was described previously (Park et al. 1992 a). The protein concentration was determined using the Biorad Microassay (Biorad, München, Federal Republic of Germany). The purity of the protein isolates was analyzed by SDS/PAGE using Phastgel Homogenous 12.5% and the Phastsystem separation and development unit (Pharmacia). Enzyme samples were treated with 2% (mass/vol.) SDS in the presence of 5% (vol.) 2-mercaptoethanol and heated at 95 °C for 2 min.

For the determination of the activity of the NADH oxidase, a suitable amount of enzyme was incubated in a 50 μl solution containing 2.4 mM FAD for 5 min at room temperature. After adding 0.9 ml of 50 mM potassium phosphate, pH 7.2, the reaction was initiated by the addition of 50 μl 3.6 mM β-NADH. Oxidation of NADH was assayed by measuring the initial decrease in the absorbance at 340 nm (absorption coefficient of 6.22 × 10³/M/cm) on the recording spectrophotometer (Shimadzu UV-160; Shimadzu, Kyoto, Japan). One unit of enzyme activity was defined as 1 μmol NADH

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oxidized/min (Cocco et al. 1988).

E. coli strains HB101 (Boyer and Roulland-Doussieux 1969), JM109 (Yanisch-Perron et al. 1985), and SG13009 (Gottesman et al. 1981) were grown in Luria-Bertani nutrient medium (Miller 1972). A genomic library of T. thermophilus HB8 in the cosmid pHC79 (Hohn and Collins 1980) has previously been constructed (Weisshaar and Sprinzl 1989). Plasmids pUC18 (Yanisch-Perron et al. 1985) and pKK223-3 containing the tac promoter (Pharmacia) were used for cloning, sequencing and expression, respectively. Plasmids pQE13 and pQE16 inserting 6 histidines to the N- or C-terminus of the expressed protein, respectively, plasmid pREP4 and immobilized Ni\textsuperscript{2+} nitrilotriacetate agarose (Ni\textsuperscript{2+}-NTA-agarose) for affinity chromatography were obtained from Diagen (Düsseldorf, Federal Republic of Germany). All other procedures used for cloning, sequencing and overexpression of T. thermophilus NADH oxidase in E. coli were described in Park et al. (1992 b).

Purification of the cloned T. thermophilus NADH oxidase from E. coli

Frozen cells were suspended (500 ml buffer per 100 g cell paste) in 50 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl, 5 mM EDTA, 100 &mu;M phenylmethanesulfonyl fluoride and 1 mM 2-mercaptoethanol at 4 °C. The cell suspension was treated with lysozyme (50 mg per 100 g cells). The cell lysate was treated with DNase I (5 mg per 100 g cells) after addition of MgCl\textsubscript{2} to an end concentration of 25 mM. The lysate was further stirred until the suspension became fluid (about 30 min). The cell homogenate was centrifuged at 12,000 g for 30 min at 4 °C to remove cell debris. The resulting cell-free supernatant was subjected to thermal treatment at 68 °C for 1 h and centrifuged at 12,000 g for 30 min at 4 °C to recover the supernatant containing heat-stable proteins.

Holoenzyme isolation

The NADH oxidase holoenzyme containing the FAD cofactor was prepared only from E. coli clones containing the respective cloned gene. The heat-treated supernatant was directly applied to a Blue Sepharose C1-6B affinity column (20  2.5 cm, total gel volume 100 ml) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl. The homogenous NADH oxidase (as demonstrated by SDS-PAGE) was eluted in the form of the holoenzyme with the same buffer containing 1 mM NADH.

Apoenzyme isolation

The heat-treated supernatant was treated slowly with ammonium sulfate to 60% saturation at 0 °C and stirred for 2 h or overnight. The protein precipitate, recovered by centrifugation at 10,000 g for 30 min, was resuspended to a protein concentration at 5 mg/ml in 50 mM sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl. The protein suspension was dialysed at 4 °C first against 5 1 of the same buffer and then against 25 mM sodium acetate buffer, pH 5.5

Fig. 1. Sequence of the nox gene encoding the NADH oxidase from T. thermophilus HB8. The amino acid sequence deduced from the gene is given by single-letter code below the nucleotide sequence. The underlined portion shows the amino acid sequence, as identified by sequencing of the N-terminal amino acids of the purified NADH oxidase from T. thermophilus HB8. Accession number of the nox gene in the GenEMBL database is X60110.