Note

Thermal Disassembly of Pyruvate Dehydrogenase Multienzyme Complex from Bacillus stearothermophilus

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Thermostabilities of component enzymes in the pyruvate dehydrogenase complex from Bacillus stearothermophilus decreased in the order lipoamide dehydrogenase, lipoate acetyltransferase, and pyruvate decarboxylase (E1). Fluorescence of an extrinsic 8-amino-1-naphthalenesulfonate (ANS) increased with inactivation of E1. The thermal denaturation of the enzymes resulted in disassembly of the complex. E1 was involved in a resulting aggregate of the complex. The interaction between ANS and denatured E1 accounted for an increase in fluorescence.

Pyruvate dehydrogenase (PDH) is a multienzyme complex catalyzing an acetyl transfer from pyruvic acid to CoA. The complex is a noncovalent assembly composed of multiple copies of three enzymes: pyruvate decarboxylase (E1, EC 1.2.4.1); lipoate acetyltransferase (E2, EC 2.3.1.12); and lipoamide dehydrogenase (E3, EC 1.6.5.3).

E1 from Bacillus stearothermophilus consists of two polypeptides, E1x and E1b.5 E1 and E3 subunits are assembled around an icosahedral core composed of two polypeptides and form the complex with its molecular size of about ten megadaltons.6 The thermostability of a protein has been extensively investigated.5 However, information on the thermal disintegration of macromolecular assemblies with large numbers of protein molecule is limited. In this study, we examined the thermostabilities of the component enzymes of PDH from B. stearothermophilus and a disassembly accompanied by its thermal denaturation.

PDH was purified from frozen cell paste of B. stearothermophilus, NCA 1503, by the method previously reported with minor modifications.8 The purification procedure included ammonium sulfate fractionation and gel filtrations on Cellulofine GCL-200-m, Sepharose CL-2B, and Sephacryl S-500HR columns. The activity of E2 was measured by the method of Schwartz and Reed.9 Overall, E1, and E3 activities were measured by the method previously reported.9 All assays were done at 30°C and pH 7. The sedimentation coefficient of purified PDH was measured at 20°C and 10,000 rpm in a Beckman An-60 Ti rotor with a Beckman Optima XL-A analytical ultracentrifuge (Fullerton, CA, U.S.A.) by the sedimentation velocity method.10 Purified PDH had an overall activity of 76.7 nkat/mg and a sedimentation coefficient of 74S. Morphological homogeneity of the PDH was confirmed by electron microscopy (data not shown). This preparation was used throughout this study. Unless otherwise noted, the buffer used was 20 mM sodium phosphate (pH 7) containing 2 mM EDTA and 0.15 mM phenylmethylsulfonyl fluoride (PMSF). Heat-treatment was done by including PDH solution at various temperatures for 30 min in a Taitec TAL-1B dry thermostat (Tokyo, Japan), and the solution was cooled in an ice bath. Sodium 8-anilino-1-naphthalenesulfonate (ANS) was measured using a molar absorption coefficient of 5000 M⁻¹ cm⁻¹ at 350 nm.11 Fluorescence at 460 nm upon excitation at 350 nm was measured at 30°C with a Hitachi 850 fluorescence spectrophotometer (Tokyo, Japan) by the method previously reported.8

Henderson et al. reported that the overall activity of PDH is retained after an incubation for 50 min at 60°C.9 To gain further information, we examined the thermostabilities of E1, E2, and E3. As shown in Fig. 1, 76% of E2 and E3 activities were retained after incubations at 77°C and 88°C, respectively. On the other hand, E1 activity was completely lost by an incubation above 72°C, and the lost activity was never restored during further incubation for three days at 4°C. The loss of the overall activity was consistent with that of E1 activity. It was reported that, because of the instability of E1, the reconstitution of PDH dissociated into its components at acidic pH or with guanidine resulted in reactivation with low yield.12 In agreement with the report, E1 was most heat labile and denatured irreversibly. When ANS was mixed as a fluorescent probe with heat-treated PDH solution, the fluorescence of ANS increased with inactivation of E1 (Fig. 1). Decrease in the fluorescence anisotropy of intrinsic FAD in E3 was also detected (data not shown). We had reported the

Fig. 1. Thermostability of a Component Enzyme in PDH and Fluorescence Intensity of ANS

After the phosphate buffer (pH 7) containing 0.12 mg/ml PDH was incubated at various temperatures for 30 min, the residual activities of E1 ( ), E2 (○), and E3 (□) were measured at 30°C and pH 7. The activity is expressed as a percentage of the activity of intact PDH. Heat-treated solution (2 ml) was mixed with 0.2 ml of 0.246 mM ANS. The fluorescence intensity ( ) is expressed as a relative value; the intensity after the heat-treatment divided by the intensity before the treatment.

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Abbreviations: PDH, pyruvate dehydrogenase; ANS, 8-anilino-1-naphthalenesulfonate; E1, pyruvate decarboxylase; E2, lipoate acetyltransferase; E3, lipoamide dehydrogenase; PMSF, phenylmethylsulfonyl fluoride.
purification of PDH from an extreme thermophile, *B. caldotenax*,
and a decrease in fluorescence anisotropy of FAD with increasing
incubation temperature, and had suggested that the thermal
denaturation of PDH resulted in reduction of its global size.\(^{19}\)
These results implied some changes in the structure of the PDH
complex. Heart-treated PDH solution, therefore, was fractionated
by ultracentrifugation in a sucrose-density gradient and analyzed.

For ultracentrifugal fractionation, 0.5 ml of a sample solution
was overlaid in a tube onto a 12-m1 bed of 10–40% (w/w) sucrose
gradient composed of 20 mM sodium phosphate (pH 7), 2 mM
EDTA, 0.15 mM PMSF, and ultrapure sucrose (Bethesda Research
Laboratories, Gaithersberg, MD, U.S.A.). The centrifugation was
done for 5 h at 32,000 rpm and 25°C with a Beckman L8-50
ultracentrifugation and a SW41-Ti rotor. The sucrose solution was
pumped out from the meniscus side of a needle tube at 0.75 ml/min
and resolved into thirty-two fractions with an ISCO-185
fractionator (Lincoln, NE, U.S.A.). Fractions at the even number
and those at the odd number were submitted to SDS-PAGE and
fluorescence measurement, respectively. SDS-PAGE was done on
a 10% separating gel.\(^{13}\) Polypeptides were stained with Coomassie
Brilliant Blue R-250, and the density of a stained band was
measured at 600 nm with a Shimadzu CS-9000 scanner (Kyoto,
Japan). In this report, the sum of polypeptides of E1x and E1β bands
and the sum of those of E2 and E3 bands are expressed as d(E1)
and d(E2/3), respectively.

The sedimentation of component polypeptides was examined
by SDS-PAGE. As shown in Fig. 2, an intact PDH complex was
detected around the fraction No. 20; d(E1) was symmetrically
co-distributed with d(E2/3). An incubation at 60°C slightly
distorted the sedimentation pattern. The distributions of d(E1)
and d(E2/3) of PDH incubated at 65°C or 70°C centered their
peaks at No. 18 and had shoulders in the bottom side. An
incubation at 80°C made these changes greater. These results at
65–80°C suggest that the complex size is reduced, a part of the
complex aggregates, and the aggregate involves E1 preferably. An
incubation at 90°C resulted in rising tendency of d(E1) toward
the bottom side, and wide distribution of d(E2/3) from the
meniscus to the bottom (Fig. 2). It was indicated that the PDH
complex was thoroughly disintegrated at this stage and that E1
was exclusively in an aggregate of the complex. ANS was mixed
with the fractions, and its fluorescence was measured. As shown
in Fig. 2, heat-driven changes in the distribution of fluorescence
were comparable with those in the distribution of d(E1) described
above. Based on these results, it was suggested that an increase
in fluorescence was caused by an interaction between ANS and
denatured E1. ANS was found to be a useful probe to monitor
distinct changes in E1 denatured below 80°C. Recently, it was
found that, during incubation at 65°C, light-scattering decreased
and then increased, and the fluorescence of ANS increased after
a lag phase (Y. Hiromasa et al., unpublished results). We speculate
that the thermal denaturation of PDH accompanies a consecutive
disassembly and/or rearrangement of the component enzymes. In
this context, further studies are in progress.

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