Purification of Pyruvate Dehydrogenase Complex from an Extreme Thermophile, Bacillus caldolyticus, and Its Thermal Stability

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Pyruvate dehydrogenase multienzyme complex was purified from Bacillus caldolyticus. The complex was composed of four polypeptides with molecular masses of 39.8, 41.7, 53.7, and 57.5 kDa estimated by SDS–PAGE and they were presumed to be pyruvate decarboxylase (E1, dimeric), lipoate acetyltransferase (E2), and lipoamid dehydrogenase (E3) on the analogy of those from Bacillus stearothermophilus. E1 and E3 were stable at pH 5.7–10.2 and 4.5–11.3, respectively. Halves of E1 and E3 activity were abolished by incubation for 30 min at 65°C and 85°C, respectively. Loss of overall activity was principally due to inactivation of E1. Structural changes in the complex incubated at high temperature were studied by fluorescence spectroscopy. The results suggested that the thermal denaturation of the complex proceeded through at least two different steps: inactivations of E1 and E3, and the former process is accompanied by a reduction of the complex size.

There have been numerous investigations on the stability of proteins from thermophilic organisms. These studies have been directed at the properties of a protein composed of one or a few polypeptides, while only a limited number of investigations on the stability of a highly oligomeric protein complex have been reported.1-3 Pyruvate dehydrogenase is a multienzyme complex with its molecular size of several megadaltons and catalyzes an acetyl transfer from pyruvic acid to CoA:

\[
\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{CoAAS} + \text{CO}_2 + \text{NADH} + \text{H}^+.
\]

Pyruvate dehydrogenase complex (PDHC) is composed of three different enzymes: pyruvate decarboxylase (E1, EC 1.2.4.1), lipoate acetyltransferase (E2, EC 2.3.1.12), and lipoamide dehydrogenase (E3, EC 1.6.4.3). On the structural features of PDHC, it has been known that the multiple copies of E1 and E3 are bound noncovalently to a core assembly formed of E2.4-5 The PDHC from a thermophile, Bacillus stearothermophilus, is more thermostable than that from a mesophile, and the structure and post-denaturational reconstruction of the PDHC assembly have been extensively investigated.6-10 However, little attention has been directed toward the thermal denaturation of the complex. Furthermore, information on PDHC from other thermophiles is limited. To shed a further light on the stability of a highly oligomeric assembly of globular proteins, this paper deals with the purification of PDHC from an extreme thermophile, Bacillus caldolyticus, and its thermal denaturation.

Materials and Methods

**Chemicals.** Bovine pancreatic ribonuclease A (RNase A, EC 3.1.27.5) and deoxyribonuclease I (DNase I, EC 3.1.21.1) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.) and Boehringer Mannheim Yamanouchi (Tokyo, Japan), respectively. Cellulose GCL-2000 was from Seikagaku Corp. (Tokyo, Japan). Sepharose CL-2B and Sephacryl S-500HR were from Pharmacia LKB Biotechnology (Tokyo, Japan). Tryptone and yeast extract were from Difco Laboratories (Detroit, U.S.A.). NAD, NADH, and CoA were obtained from Khojin Co., Ltd. (Tokyo, Japan). Hen egg white lysozyme (EC 3.2.1.17), phenylmethanesulfonyl fluoride (PMSF), thiamine pyrophosphate (TPP), lipoamide, and 2,6-dichlorophenol indophenol (DCIP) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of the highest grade commercially available.

**Bacterial strain and growth conditions.** Bacillus caldolyticus, EP00275, was a gift from Dr. M. Kinuma, Kyushu University, Japan. The cells were cultured aerobically at 67°C in L-broth (1% Tryptone, 0.5% yeast extract, and 1% NaCl at pH 7.4). In the late exponential phase of the growth the cells were harvested by a centrifugation for 15 min at 6000 x g and stored at −32°C until use.

**Enzyme assays.** Unless otherwise noted all enzyme assays were done at 30°C with continuously monitoring of absorbance changes at a given wavelength. The monitoring was done by a Hitachi U-3210 recording spectrophotometer (Tokyo, Japan) with an Iuchi CC-301 cuvette stirrer and a CB15 circulator (Tokyo, Japan). Overall activity was measured by the method of Perham and Lowe11 with minor modifications. For an assay of the overall activity 50 μl of an enzyme solution was mixed with 50 μl of 100 mM sodium pyruvate, 50 μl of 0.15 mM CoA (containing 130 mM DTT), and 950 μl of 52 mM potassium phosphate buffer (pH 7) containing 1.05 mM magnesium chloride, 0.021 mM TPP, and 2.8 mM NAD. One unit was defined as the amount of PDHC capable of producing one μmol of NADH per min under these conditions. NADH was measured using a molar absorption coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm.12 E1 activity was measured principally by the method of Lowe et al.13 The reaction mixture for the assay consisted of 30 μl of an enzyme solution, 50 μl of 100 mM sodium pyruvate and 950 μl of 100 mM potassium phosphate buffer (pH 7) containing 1 mM magnesium chloride, 0.1 mM DTT, and 0.2 mM TPP. One unit was defined as the amount of PDHC capable of reducing one μmol of DCIP per min under these conditions. DCIP was measured using a molar absorption coefficient of 19,100 M⁻¹ cm⁻¹ at 600 nm.14

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**Abbreviations:** PDHC, pyruvate dehydrogenase complex; E1, pyruvate decarboxylase; E2, lipoate acetyltransferase; E3, lipoamide dehydrogenase; RNase A, bovine pancreatic ribonuclease A; DNase I, deoxyribonuclease I; DTT, 1,4-dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TPP, thiamine pyrophosphate; DCIP, 2,6-dichlorophenol indophenol; Iₚ, intensity of vertically polarized fluorescence; I₀, intensity of horizontally polarized fluorescence.
The E3 assay was done by the method of Reed and Willims15 with minor modifications. To 100 μl of 1 mM NADH were added 300 μl of 1 mM NAD, 30 μl of 100 μM lipoamide (in 95% EtOH) and 500 μl of 300 mM sodium 2-(N-morpholino)ethanesulfonate buffer (pH 7). The reaction was started by the addition of 50 μl of an enzyme solution, and its rate was measured by the absorbance change at 340 nm. One unit was defined as the amount of PDHC which caused oxidation of one μmol of NADH per min under these conditions.

Protein measurement. Protein was measured with a Bio-Rad protein assay kit (Tokyo, Japan) using bovine serum albumin as a standard protein.

Electrophoresis. SDS-PAGE was done on 9% separating gel by the method of Laemmli19 with a Sigma MW-SDS-200 molecular weight marker (St. Louis, U.S.A.). The protein was stained with Coomassie Brilliant Blue.

Purification of PDHC complex. Unless otherwise noted all operations were done at 4°C, and centrifugations were done for 15 min at 5000 × g. An Asahi Kasei Mini-module NM-3 ultrafiltrator (Tokyo, Japan) was used for concentrating a protein solution. All columns used for chromatographies were pre-equilibrated and developed with an elution buffer (pH 7) consisted of 20 mM sodium phosphate, 2 mM EDTA, and 0.15 mM PMSF.

PDHC was detected by measuring E1 activity.

Frozen cell paste (12.5 g, wet weight) was thawed and suspended for one hour in 28 ml of 50 mM sodium phosphate buffer (pH 7) containing 5 mM EDTA, 0.15 mM PMSF, 6 mg/ml lysozyme, and 5 μg/ml DNase I. The suspension was passed through ten consecutive 30-sec-disruptions at 60 W by a Tomy Seiko UR-200P ultrasonic oscillator (Tokyo, Japan). After the cell debris were removed by centrifugation, the supernatant was brought to 25% saturation with ammonium sulfate and stirred for 30 min. The resulting precipitate was centrifuged out, and the supernatant was brought to 55% saturation with ammonium sulfate and stirred for 30 min. The precipitate collected by centrifugation was resuspended in 20.5 ml of the elution buffer and then put on a Sepharose CL-2B column (3.3 × 92 cm).

Active fractions eluted from the column were combined and brought to 70% ammonium sulfate saturation. The precipitate was suspended in 8.2 ml of the elution buffer and chromatographed twice on a Cellulofine GCL-2000-m column (1.4 × 68 cm). Combined active fractions were concentrated and chromatographed on a Sephacryl S-500HR column (1.5 × 113 cm). Active fractions eluted from the Sephacryl column were rechromatographed three times. After the last column chromatography, active eluents were combined, concentrated to 5 ml, and incubated with 0.2 ml of an RNase A solution (83.2 μg/ml) for 2.5 h at room temperature. Finally, the mixture was rechromatographed on the Sephacryl S-500HR column, and combined active fractions were concentrated. Sodium azide was added to the enzyme solution thus prepared to yield final concentration of 0.2 mg/ml, and the preparation was stored at 4°C until use.

Kinetic analysis. The overall activity was measured with various concentrations of 2-oxoisovaleric and pyruvic acids as substrates under the conditions described above. Using the EKAS program (Mitsui Knowledge Industry, Tokyo, Japan),17,18 values of the Michaelis constant (K_m) and maximum velocity (V_max) were computed by a plot of [s]/v against [s], where [s] and v are the concentration of substrate and initial reaction rate, respectively.

pH optimum and stability. The overall activity was measured in a buffer solution at various pHs. Buffers used were 50 mM sodium citrate from pH 4.1 to 6.1, 50 mM potassium phosphate from pH 6.0 to 7.7, and 50 mM sodium borate from pH 7.6 to 10.6. The pH-stability of the PDHC was examined in the following manner. To 30 μl of enzyme solution (14–28 μg/ml) was added 950 μl of the universal pH buffer containing phosphate, acetate, and borate, which was designed to provide a constant ionic strength of 0.1 M at various pHs from 2.1 to 12.2. The incubation was done at 4°C for 25 h, and then the remaining E1, E3, and overall activities were measured spectrophotometrically by the methods described above.

Thermal stability. One ml of the enzyme solution (1.9 mg/ml) was incubated for 30 min at various temperatures from 30 to 90°C and pH 7, where the pH of solution was adjusted at 25°C. After the solution was cooled, the remaining E1, E3, and overall activities were measured by the methods described above. Time-dependent changes in E1 activity were measured using the enzyme solution diluted 1:10 in 20 mM sodium phosphate buffer (pH 7) containing 2 mM EDTA.

Fluorescence measurements. Unless otherwise noted the enzyme solution diluted 1:10 in 20 mM sodium phosphate buffer (pH 7) containing 2 mM EDTA and filtered with a 0.45 μm Dismic-3CP membrane filter (Advantec, Tokyo, Japan) was used for spectrophotometric measurements. The measurements were done with a Hitachi 850 fluorescence spectrophotometer (Tokyo, Japan) with a homemade cuvette stirrer and an Iuchi CB15 circulator. For measuring the anisotropy of fluorescence a Hitachi 650-1572 polarizer unit was used additionally. The fluorescence intensity of the enzyme solution (40 μg/ml) excited at 290 nm was measured at various temperatures from 20 to 70°C. The intensity was converted into the quantum yield using N-acetyl-tryptophanamide as the standard.20 Time-dependent changes in the fluorescence intensity and anisotropy were examined in the following manner. The excitation wavelength was set at 450 nm. After the enzyme solution (82 μg/ml) was incubated at 70°C for various times, the emission spectrum of the solution thermostatted at 70°C was monitored from 500 to 550 nm. For the measurement of anisotropy at 520 nm, the vertically polarized light at 450 nm was used to irradiate the enzyme solution and, intensities of vertically and horizontally polarized fluorescences, I_v and I_h, were monitored at 70°C. The anisotropy, r, was evaluated from the expression: \( r = (I_v - I_h)/(I_v + 2I_h) \).21 Effects of incubation temperature on the fluorescence anisotropy was examined using the enzyme solution (82 μg/ml), was cooled immediately after incubation for 30 min at various temperatures. The anisotropy was estimated in the same manner as described above except that the polarized fluorescences were monitored at 30°C. The lifetime of fluorescence at 520 nm was measured at 30°C with a Horiba NAES-1100 time-resolved spectrofluorometer (Tokyo, Japan) upon excitation at 450 nm.

Results and Discussion

(1) Purification of PDHC and its properties

PDHC complex was purified from the cell-free extract of B. caldolyticus by ammonium sulfate fractionation followed by gel filtration column chromatographies. As summarized in the Table, 32.5 units (in terms of overall activity) of PDHC was obtained from 12.5 g of the frozen cells, and

Table: Purification Procedure of Pyruvate Dehydrogenase Multienzyme Complex from Bacillus caldolyticus

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Overall activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (U/mg)</th>
<th>E1 activity (U)</th>
<th>E3 activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>531.5</td>
<td>99.0</td>
<td>0.19</td>
<td>100</td>
<td>21.7</td>
<td>450.2</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>387.3</td>
<td>74.3</td>
<td>0.19</td>
<td>75.1</td>
<td>13.6</td>
<td>392.3</td>
</tr>
<tr>
<td>Sepharose CL-2B</td>
<td>110.6</td>
<td>96.5</td>
<td>0.87</td>
<td>97.5</td>
<td>18.4</td>
<td>523.0</td>
</tr>
<tr>
<td>Cellulofine GCL2000</td>
<td>82.2</td>
<td>60.5</td>
<td>0.74</td>
<td>61.1</td>
<td>8.5</td>
<td>269.2</td>
</tr>
<tr>
<td>1st Sephacryl S500</td>
<td>8.7</td>
<td>40.2</td>
<td>4.6</td>
<td>40.8</td>
<td>5.6</td>
<td>126.6</td>
</tr>
<tr>
<td>5th Sephacryl S500</td>
<td>6.0</td>
<td>32.5</td>
<td>5.4</td>
<td>32.8</td>
<td>4.3</td>
<td>76.4</td>
</tr>
</tbody>
</table>

* Calculated from the total protein and overall activity.
* Calculated for the overall activity.
* Activity of the pyruvate decarboxylase.
* Activity of the lipoamide dehydrogenase.
the preparation contained 4.3 units of E1 and 76.4 units of E3. The Table also indicates that the activity ratios (E1)/(overall) and (E3)/(overall) in the purified preparation are 50–60% of those in the crude homogenate. This may imply the existence of some contaminants affecting enzyme activities in the crude homogenate and/or changes in the protein composition of the complex during the purification. However, further studies were not done on this. From the results of SDS–PAGE analysis, the PDHC was found to be composed of four polypeptide chains with apparent molecular masses of 39.8, 41.7, 53.7, and 57.5 kDa. It has been reported that the PDHC from a Gram-positive bacterium consisted of four polypeptides E1α, E1β, E2, and E3 and the molecular sizes of the polypeptides were quite similar to those of PDHC from B. stearothermophilus and B. subtilis. Although detailed assignments of the four bands detected on an SDS–polyacrylamide gel were not done, it was assumed that the PDHC from B. caldolyticus was also composed of E1α, E1β, E2, and E3 subunits. From kinetic analysis of the overall activity, it was found that pyruvate was a better substrate for the complex than 2-oxoisovalerate: $K_m$ and $V_{max}$ for pyruvate were $0.117 \pm 0.03 \text{mM}$ and $15.4 \pm 0.266 \text{nmol s}^{-1}$, respectively, and those of $K_m$ and $V_{max}$ for 2-oxoisovalerate were $1.27 \pm 0.22 \text{mM}$ and $4.52 \pm 0.27 \text{nmol s}^{-1}$, respectively. Based on these results, pyruvic acid was used as the substrate for PDHC throughout these experiments. As shown in Fig. 1, the overall activity at 30°C was found to be maximal at pH 7.5 to 8. The reason why the activity in the borate buffer was lower than in the phosphate buffer remains unknown. E1 activity at 70°C was highest at pH 7 (data not shown). Without detailed data including the purification method of PDHC, Visser et al. have stated for PDHC of B. caldolyticus that molecular weights of the four component polypeptides were 37, 40–41, 53, and 58–59 kDa, the optimum pH of the enzyme at 55°C was 7 to 7.4, and optimum temperature was at 70°C.

Their descriptions substantially support our results.

(2) Stability of PDHC

After the incubation of PDHC for 25 h at various pHs and 4°C, the overall, E1, and E3 activities were measured. As shown in Fig. 2, more than 80% of the overall and E1 activities were preserved between pH 6 and 10, but most parts of these activities were lost below pH 4 and above pH 11. On the other hand, above 80% of E3 activity remained between pH 4.5 and 11.3. Furthermore, after incubation for 30 min at various temperatures, the remaining E1, E3, and overall activities at 30°C were assayed at pH 7. As shown in Fig. 3, all of these activities remained unchanged after the incubation at 60°C. However, the incubation at 70°C resulted in marked losses of the overall and E1 activities. In contrast to this, no significant loss of the E3 activity was observed after the incubation below
85°C, and the activity was abolished by the incubation at
90°C. From these results, it is shown that pH and thermal
stabilities of PDHC depend on those of E1, and that E3 is
much more stable than E1. It has been reported that the
E2–E3 core assembly in PDHC from *B. stearothermophilus*
was more stable than E1 and capable of reassociation after
dissociation by several denaturants.8) These and our results
imply similarity of the stabilities between PDHCs purified
from both thermophiles.

(3) Fluorescence spectroscopic studies on thermal denatura-
tion of PDHC

Fluorescence spectroscopic studies were done to discover
the thermal denaturation mechanism of PDHC. Upon
excitation at 290 nm, a fluorescence band of PDHC with a
maximum at 330 nm was observed. From excitation and
emission spectra this emission was ascribed to the fluo-
rescence of typtophan residues in PDHC. This fluorescence
emission was quenched with increasing temperature
(inset in Fig. 4) and this fluorescence quenching accom-
panied a red shift of the peak position. The quantum yield
Φ is defined as Φ = k_r/(k_r + k_a), where k_r and k_a are the
rate constants of radiative and non-radiative transition
from the excited singlet state, respectively. k_r is ordinarily
based on an electronic transition and, therefore, in-
dependent of the temperature, while k_a is caused by the
interactions between a tryptophan residue and its
environments, and its temperature dependence is re-
presented by k_a = A_0 exp(−ΔE/RT). The temperature
dependence of quantum yield is therefore, expressed as

\[ \ln(1/\Phi - 1) = \ln(A_0/k_r) - \Delta E/RT \]  (1)

When the fluorescence intensity was converted into the
quantum yield and plotted at various temperatures based
on Eq. 1, it gave two straight lines intersecting at around
60°C (Fig. 4). These results showed that the interaction
mode of tryptophan residues with their surroundings
changed critically at this temperature.

When PDHC was excited at 450 nm, a very weak but
clear emission with a maximum at 520 nm was recognized.
Since E3 is generally known to be an FAD-containing
flavoprotein, the emission was ascribed to the fluorescence
of FAD. At 70°C, time-dependent changes in the emission
intensity and fluorescence anisotropy were measured. E1
activity was also examined immediately after various
incubation times at this temperature. Within the incubation
time of 20 min, the intensity increased by a factor of 1.2,
the value of anisotropy was reduced to 0.27, and E1 activity
was abolished (Fig. 5). The lost activity was not recovered
after standing for 24 h at 4 or 25°C. Since the fluorescence
anisotropy decreases with increasing fluorescence life-
time, 1) the lifetime was measured. The fluorescence lifetimes
at 520 nm before and after the heat treatment for 30 min at
70°C were evaluated to be 87.6 ± 11.4 and 63.3 ± 9.2 s.

![Fig. 4. Temperature Dependence of Fluorescence Intensity at 330 nm.](image)

Relative fluorescence intensities of PDHC solutions were measured at temperatures indicated (Inset). The data were replotted as \( \ln(1/\Phi - 1) \) vs. \( 1/T \), where \( T \) and \( \Phi \) are absolute temperature and quantum yield of the fluorescence, respectively (see Materials and Methods).

![Fig. 5. Time-Dependent Changes in E1 Activity, Fluorescence Intensity and Its Anisotropy at 520 nm.](image)

After the indicated times of incubation at 70°C, the relative fluorescence intensity (Δ) and fluorescence anisotropy (○) were measured at the temperature. E1 activity (●) was at 30°C after the incubation (see Materials and Methods).

![Fig. 6. Effects of Incubation Temperature upon Anisotropy of Fluorescence at 520 nm.](image)

After incubations of PDHC solution for 30 min at temperatures indicated, the fluorescence anisotropy was measured at 30°C (see Materials and Methods).
respectively. It was, therefore, indicated that the decrease of fluorescence anisotropy was not caused by the variation of fluorescence lifetime. Moreover, effects of the incubation temperature on the fluorescence anisotropy at 520 nm were studied. As shown in Fig. 6, the anisotropy of 0.35 was almost constant independent of the temperatures up to 60°C. With increasing temperature, the anisotropy greatly decreased and gave 0.1 at the incubation at 90°C. Through Perrin-Weber equation, which gives the relationship between the anisotropic value of steady-state fluorescence and the rotational correlation time of the fluorophore, the decrease of fluorescence anisotropism observed above 60°C suggests that the size of PDHC components containing E3 is reduced and the reduction closely correlates with the inactivation of E1. Although the fluorescence maximum position of PDHC (330 nm) implies that the tryptophan residues are in the hydrophobic region of this protein, it is clear from the spectral red shift that the heat treatment changes the circumstances of these residues. Furthermore, the detailed analysis of the temperature dependence of the tryptophan fluorescence showed the local conformation around the tryptophan residue was altered critically at 60°C. These spectroscopic studies consistently show that heat treatment results in some degradation of the global structure of PDHC.

After incubation at various temperatures, a sample of PDHC solution was analyzed by the non-denaturing electrophoresis on a 0.6% agarose gel. The PDHC solution incubated above 70°C was stained as conspicuous broad and smeared bands. When the PDHC from B. stearothermophilus was thermally denatured at 80°C, wide distribution of various molecular species was observed in the ultracentrifugal field (unpublished). These observations makes the thermal denaturation of the PDHC worthy of further study.

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References