Thermally Induced Disintegration of the *Bacillus stearothermophilus* Dihydrolipoamide Dehydrogenase†

Yasuaki Hiromasa, Yoichi Aso,*,†† Shoji Yamashita,** and Kohji Meno*

RIKEN, Institute of Physical and Chemical Research, Sayo-gun, Hyogo 679-5143, Japan

*Laboratory of Protein Chemistry and Engineering, and ** Laboratory of Biophysics, Kyushu University, Fukuoka 812-8581, Japan

Received March 17, 2000; Accepted May 9, 2000

Upon heat treatment of the pyruvate dehydrogenase complex from *Bacillus stearothermophilus*, the most thermostable component is a dihydrolipoamide dehydrogenase (E3c). To understand this stability, the thermal disintegration of E3c dissociated from the complex (E3d) was examined, comparing that of E3c. Judging from residual activity and inactivation rate, E3d was less thermostable than E3c; E3d and E3c lost half of their original activities upon incubations for 30 min at 79°C and 90°C, respectively. Heat treatment of E3d raised the fluorescence intensities of Trp residue, intrinsic FAD, and extrinsic 8-anilino-1-naphthalene-1-sulfonate. E3d lost FAD, and inactive E3d polypeptides were aggregated. The sulfonate bound to the aggregate became notably fluorescent. The thermal disintegration of E3d was speculated to be a consecutive reaction that was different from the concurrent disintegration reaction of the complex. Some interactions with other component polypeptides was suggested to improve the thermostability of E3c.

Key words: *Bacillus stearothermophilus*; dihydrolipoamide dehydrogenase; pyruvate dehydrogenase complex

Pyruvate dehydrogenase is a multienzyme complex catalyzing the acetyl transfer from pyruvate to CoA: the synthesis of an acetyl CoA. The *Bacillus stearothermophilus* pyruvate dehydrogenase complex (PDC) is one of the well characterized complexes, and its component enzymes are pyruvate decarboxylase [EC 1.2.4.1] (E1), lipoate acetyltransferase [EC 2.3.1.12] (E2), and dihydrolipoamide dehydrogenase [EC 1.8.1.4] (E3).1–4 These components not only have different functions, but also make different contributions to the construction of PDC. E1 and E2 facilitate the transfer which results in the reduction of a lipoyl group covalently attached to the lipoyl domain of an E2 polypeptide, and E3 facilitates the oxidation of the resulting dihydrolipoyl group using the conversions of FAD to FADH₂ and of NAD⁺ to NADH. The backbone structure of PDC is an assembly of sixty homo-polypeptides of E2, and either hetero-tetrameric E1 or homo-dimeric E3 binds to the binding domain of an E2 polypeptide. PDC thus comprises thirty (E1α)₂(E1β)₂, six (E3)₆, and an (E2)₆0.2–4 The stability of the component enzymes is also different from one other. It is possible to measure the enzyme activity of a component independently of other components; e.g., the E3 activity can be measured with free dihydrolipoamide in the absence of E2. Judging from changes in activity thus measured, E3 is the most thermostable component.5–7 It was however unclear whether this stability is only due to a rigidity of E3, because a prolonged incubation at high temperatures yields a few protein assemblies in which active E3 is incorporated. Knowledge of the thermostability of E3 in the absence of other components will help us to obtain better understanding of the high stability of E3 in PDC. Since the dimeric and active E3 dissociated from PDC is available, we have embarked on the examination of temperature-induced changes in E3 dissociated from PDC and compared with those of E3 in PDC; E3d and E3c refer to the dissociated E3 and the E3 in PDC, respectively, in this report.

Materials and Methods

Chemicals and enzymes. All reagents used were of the highest grade commercially available. Unless otherwise noted, the buffer used was 20 mM sodium phosphate buffer (pH 7) containing 2 mM EDTA and 0.15 mM phenylmethanesulfonyl fluoride; this is

---

† This work was supported in part by the Special Postdoctoral Researchers Program of RIKEN, Japan.

†† To whom correspondence should be addressed. FAX: 81-92-642-3051; E-mail: yaso@agr.kyushu-u.ac.jp

Abbreviations: ANS, 8-anilino-1-naphthalene-1-sulfonate; E1, pyruvate decarboxylase; E2, lipoate acetyltransferase; E3, dihydrolipoamide dehydrogenase; E3c, E3 in pyruvate dehydrogenase complex; E3d, E3 dissociated from pyruvate dehydrogenase complex; PDC, pyruvate dehydrogenase complex
referred to as buffer P. FAD and 8-anilinonapthalene-1-sulfonate (ANS) were obtained from Sigma (Tokyo) and Nacalai Tesque (Kyoto), respectively. Superoxide 12HR10/30 column was purchased from Pharmacia LKB Biotechnology (Tokyo). Diaphorase II (lyophilized) from *B. steaothermophilus* (NCA 1503) was purchased from Unitika (Kyoto) and further purified by ion-exchange chromatography by the following method. The sample (2 mg) was dissolved with 2–3 ml of buffer P and put on a Pharmacia HiTrap-Q column (2 ml) equilibrated with the same buffer. After the column was washed with buffer P, proteins were eluted by a linear gradient of NaCl from 0.0 to 2.5 m in the buffer. Active fractions were combined, dialyzed against buffer P for 48 h, and used as E3d. The purity of E3d thus prepared was confirmed by SDS-polyacrylamide gel electrophoresis. PDC was purified from the frozen cell paste of the *B. steaothermophilus* by the method previously reported and used as E3c.6)

**Protein and FAD measurements.** Protein was measured with a BioRad protein assay kit (Tokyo, Japan) using bovine serum albumin as standard protein. The amount of FAD in a protein was fluorometrically measured using free FAD as a standard by the method previously reported.6)

**Heat treatment.** Unless otherwise noted, a heat treatment was done by the following method. The buffer P (0.05 ml) containing an enzyme was added to the buffer P (0.95 ml) thermostatted at a given temperature. The mixture was incubated at the temperature for 30 min and cooled on ice.

**Assay of enzyme activity and kinetic analysis.** Enzyme activity was measured at 30°C and pH 7 by the method reported previously.5,6) Briefly, the oxidation of a dihydrolipoamide was evaluated by spectroscopic measurement of the conversion of NAD+ to NADH. The specific activities of E3d and E3c were measured using 61.4 μg/ml E3d and 180 μg/ml PDC, respectively. Rate constants of various reactions were evaluated by an exponential fit on the assumption of first order kinetics; experimental data used were those in an initial reaction phase. Kinetic parameters of E3d (0.390 mg/ml E3d) and E3c (0.215 mg/ml PDC) were measured using 0.0156–0.313 mm dihydrolipoamide as a substrate and plots of [S]/V vs. [S].6)

**Spectroscopic measurements.** All spectroscopic measurements were done at 30°C with a Hitachi 650–60 fluorescence spectrophotometer. Fluorescence spectra ascribed to FAD, Trp residue, and ANS were measured upon excitations at 450 nm, 295 nm, and 350 nm, respectively. For the measurement of ANS fluorescence, 1.0 ml of 24 μM ANS was added to 0.2 ml of a test solution. Light scattering at a right angle was measured with the fluorescence spectrophotometer by setting both the excitation and emission wavelengths at 600 nm.

**Gel filtration.** The gel filtration was done at 20°C on a Superoxide 12HR column (1.0 × 30 cm) equilibrated with buffer P, using a Tosoh 8020 HPLC system.

**Results**

**Changes in enzyme activity**

For E3d and E3c without heat treatment, we measured the following activities: the E3 activities per unit amount of protein (U/mg) and per unit amount of FAD (U/nmol). The activities of E3d were 32.0 U/mg and 2.30 U/nmol. The activities of E3c were 8.30 U/mg and 4.07 U/nmol. We examined $V_{\text{max}}$ and $K_m$ of intact E3d and E3c using 0.0156–0.313 mm dihydrolipoamide as a substrate. The $V_{\text{max}}$ values of E3d and E3c were 0.936 and 2.41 mm/min/nmol, respectively; $V_{\text{max}}$ was evaluated as the maximum velocity per unit amount of FAD. The $K_m$ values of E3d and E3c were 84.6 and 193 μM, respectively. Using these data, the $V_{\text{max}}/K_m$ values of E3d and E3c are calculated to be 11.1 and 12.5/min/nmol, respectively.

A heat treatment at high temperature resulted in irreversible loss in enzyme activity. An incubation at 4°C for several days after the heat treatment never restored lost activity. The inactivations of E3d and E3c were exponentially dependent on time at least in an initial reaction phase, and their rates increased with temperature (Fig. 1A & 1B). E3d was clearly less stable than E3c. Upon heat treatment at 86°C, the rate constants of E3d and E3c inactivations were evaluated to be 4.42 × 10⁻²/min and 3.49 × 10⁻³/min, respectively. Furthermore, E3d and E3c lost halves of their original activities after heat treatments at 79°C and 90°C, respectively (Fig. 1C). On the basis of the Arrhenius relationship between inactivation rate constant and temperature, the activation energies for E3d and E3c inactivation reactions were calculated to be 473 kJ/mol and 388 kJ/mol, respectively. The rate constant of the inactivation reaction of E3d at 83°C was almost independent of protein concentration; its value varied from 4.10 × 10⁻⁵ to 5.61 × 10⁻²/min at concentrations between 78.0 and 564 μg/ml. On the other hand, the rate constant of the inactivation reaction of E3c at 92°C decreased with increasing concentration of PDC from 59.8 to 395 μg/ml (Fig 1D).

**Changes in fluorescence spectrum**

After heat treatment of E3d at various temperatures, we measured the fluorescence spectra ascribed to the Trp residue, intrinsic FAD, and ANS added after the treatment. The temperature was elevated to
Thermal Disintegration of Dihydrolipoamide Dehydrogenase

Fig. 1. Temperature-induced Inactivations of E3d and E3c.
Panel A. E3d (30.5 µg/ml) was incubated at 78°C (●), 80°C (○), 83°C (▲), and 86°C (△) for a given time. Panel B. PDC (253 µg/ml) was incubated at 86°C (○), 89°C (●), 92°C (▲), and 95°C (△) for a given time. Panel C. E3d (218 µg/ml; ●) and PDC (222 µg/ml; ○) were incubated for 30 min at a given temperature. Panel D. PDC (395 µg/ml, ○; 215 µg/ml, ▲; 59.8 µg/ml, △) were incubated for a given time at 92°C. After these heat treatments, the residual activity of E3 was measured at 30°C and is plotted by taking the corresponding activity of an intact E3 as 100%. Solid lines in panels A and B are time-courses made by fitting experimental data to an exponential decay curve.

Fig. 2. Temperature-induced Changes in Fluorescence Intensities of Trp Residue (A & D), FAD (B & E), and ANS (C & F).
Panels A-C. E3d (282 µg/ml) was incubated at 78°C (●), 83°C (○), and 86°C (▲). Panels D-F. E3d (209 µg/ml) was incubated for 30 min at various temperatures. After these heat treatments, the fluorescence intensities at 30°C of Trp residue (A & D), FAD (B & E), and ANS (C & F) were measured at 340 nm, 520 nm, and 460 nm, respectively. The intensities are plotted as relative values by taking the corresponding intensity of intact E3d as one.

30°C for measurement as described in Materials & Methods, but we confirmed that the spectra change insignificantly within a measuring time. A heat treatment for 30 min caused similar changes in fluorescence spectrum. The emission maximum of Trp fluorescence of intact E3d was at 340 nm and shifted to 333 nm after treatment at temperatures above 70°C. The emission maximum of FAD fluorescence of intact E3d was at 535 nm and shifted to 526 nm after treatment at temperatures above 83°C. Upon mixing ANS with an intact E3d, the emission maximum of ANS fluorescence was at 484 nm and shifted to 471 nm after treatment at temperatures above 86°C. The intensity of fluorescence increased, depending on time and treatment temperature (Fig. 2). The heat treatment at temperatures between 83°C and 92°C for 30 min raised the fluorescence intensities of Trp at 340 nm, FAD at 520 nm, and ANS at 460 nm 10-times, 8-times, and 29-times, respectively, as high as the corresponding intensity of intact E3d (Fig. 2D-2F). At temperatures ranged from 60°C to 86°C, increase in fluorescence intensity as a function of temperature was well correlated to each other; especially, the correlation coefficient between increasing ratios of FAD and Trp fluorescence intensities was calculated to be 0.9996.

Upon treatment at 83°C, the changes in intensities of Trp and FAD fluorescence were independent of protein concentration (Fig. 3A-3B), but those of ANS fluorescence was slightly dependent on the concentration (Fig. 3C). For Trp and FAD fluorescence, the values of first-order rate constants were similar to each other and insignificantly dependent on protein concentration (39.0–564 µg/ml); the average rate constants for changes in fluorescence intensities of FAD and Trp were calculated to be 7.47 × 10⁻⁷/min and 8.63 × 10⁻⁷/min, respectively. The fluorescence anisotropy of non-treated E3d was 0.32, but it was reduced to 0.28 and 0.08 by heat treatments at 65°C and 83°C, respectively. Heat treatment increased the
light scattering of an E3d solution; the increase became faster with increasing temperature (data not shown) and was slightly dependent on protein concentration (Fig. 3D).

To obtain further information on changes in molecular state of FAD, we examined the effects of heat treatment on the following two solutions: free FAD (7.64–1.53 μM) and E3d containing similar amounts of FAD (7.50–1.50 μM). The FAD fluorescence intensity of both solutions was measured before and after incubation at 83°C for 30 min; the value of the intensity was corrected to that per unit amount of FAD. There was insignificant difference between fluorescence intensities of the free FAD solutions before and after incubation. The fluorescence intensity of the E3d solution was about one-fifth of that of the free FAD solution before incubation and raised to 75–90% of the free FAD solution after incubation. We confirmed that neither EDTA nor PMSF in the buffer was responsible for an increase in FAD fluorescence of E3d.

Changes in molecular form

E3d incubated at various temperatures for 30 min was resolved by gel filtration. As shown in Fig. 4, three peaks, P-I, P-II, and P-III, of the absorbance at 280 nm were detected. Inactive and active E3d's were detected in P-I and P-II, respectively. FAD was detected in P-II and P-III. Appreciable amounts of protein were not detected in P-III. An unidentified peak at a similar position to that of P-III was detected upon gel filtration of an intact E3d; the fractions around this peak contained neither FAD nor polypeptide. With increasing temperature, the heights of P-I, P-II, and P-III increased, decreased, and increased, respectively; finally, P-II was completely converted into P-I and P-III (Fig. 4). To obtain more information on the molecular state of FAD, E3d treated at 83°C was dialyzed against the buffer P at 4°C for 48 hr, and the amount of FAD that remained in a dialyzing bag was measured. As a control, the same experiment was done for free (unbound) FAD. In this control experiment, the residual amount of FAD was under the detection limit. On the other hand, 11% of original FAD in E3d remained after heat-treatment of E3d and successive dialysis.

To know the molecular species responsible for changes in fluorescence intensity, E3d incubated at 83°C for 10–40 min was filtered on a gel, and the distribution of fluorescence and FAD amount of eluate were examined. Three peaks of fluorescence were detected; both the peaks of Trp and ANS fluorescence were in fraction No. 5 (Fig. 5A & 5B), and those of FAD fluorescence were in No. 7 and 11 (Fig. 5C). The two peaks of FAD amount were also at No. 7 and 11 (Fig. 5D). Considering differences in volume of a fraction, the peaks at No. 5, 7, and 11 corresponded to P-I, P-II (F-I), and F-II, respectively (Fig. 5 & 6). With incubation time, the intensities of Trp, FAD, and ANS fluorescence increased at the corresponding peak position, and the elution position of FAD changed from fraction No. 7 to 11 (Fig. 5).

Discussion

At the outset we examined the difference in enzyme activity between E3d and E3c. Based on protein amount the specific activity of E3d was four times as high as that of E3c, but is less than estimated on calculation, because the total number of polypeptides comprising PDC is sixteen times as many as the number of E3c polypeptides. The specific activity based on its molar concentration can be evaluated for E3d, but such an activity is not easily available for E3c, because the measurement of the E3 content in PDC besides that of the molecular weight of PDC is quite difficult.2–4) Assuming that there are six E3 dimers in a molecule of 9 MDa PDC,9) however, we estimated the specific activity of E3c (U/mol E3) roughly. Results indicated that the specific activity of E3d is 31% of that of E3c. Since the FAD molecule binds solely to E3 polypeptide, the comparison based on FAD contents measured is expected to clarify the difference in E3d and E3c activities. The E3d activity thus evaluated was 57% of E3c activity. The value of $V_{max}$ of E3d was 40% that of E3c. On the other hand, the $K_{m}$ value of E3d was less than that of E3c, and then, the difference in the values of $V_{max}/K_{m}$ was slight.

Based on the results from analysis of crystal structure.
of a dimeric E3 complexed with its binding domain (fragment) of an E2 polypeptide, it is reported that the complex formation induces some steric changes in the active site region of E3, although the global conformation of E3c is quite similar to that of E3d.\(^3\)

We deduced the following speculation from these results. The dissociation of E3 from PDC, namely a change in molecular state from E3c to E3d, raises the affinity of a substrate to the enzyme, but reduces the reaction rate. These effects compensate for each other and induce little differences in catalytic efficiency between E3d and E3c. However, the dissociation of E3c might decrease the activity even when it is not accompanied by denaturation of E3. We reported that, upon treatment of PDC with potassium iodide at high concentrations, the decrease in E3 activity is more notable than that in FAD content.\(^5\) It is likely that the difference in activities between E3d and E3c is partly responsible for this result. Both the E3d and E3c lost their activities irreversibly by incubation at elevated temperatures, but the relationship between incubation temperature and residual activity was clearly different. Based on differences in activity after incubation for a given time and those in inactivation rate at a given temperature, E3d was less heat-resistant than E3c. The rate constant of the inactivation reaction of E3d was insignificantly dependent on protein concentration, suggesting that the reaction proceeds spontaneously without protein-protein interaction. The gel filtration analysis of heat-treated E3d indicated the conversion of active E3d to aggregate(s) exclusively composed of inactive E3d. Based on these results it was concluded that E3d’s are aggregated after their inactivation, and the aggregation might be responsible for the irreversibility of the inactivation reaction. On the other hand, the rate constant of the inactivation reaction of E3c decreased slightly with increasing concentration of PDC. This was more notable at the late phase of inactivation than at the initial phase. We previously reported that the incubation of PDC at high temperature never results in a simple dissociation into its components.\(^6\) After prolonged heat treatment, E3 activities are detected in at least two molecular species: an E1-rich aggregate(s) larger than PDC and an E2-rich complex smaller than PDC.\(^6\) Both E3’s in these species have slightly higher specific activity (units/FAD) than that of intact E3c. Based on these results, we speculate on the reason why the thermostability of E3c is higher than that of E3d as follows: it is not only because some interactions between E3 polypeptide and its binding domain of E2 polypeptide stabilize E3c, but also because changes in molecular species to which E3c belongs raise the E3 activity slightly. The protein-concentration dependency of the inactivation reaction rate of E3c seems to be due to the latter reason. The comparison between the temperature-driven inactivations of E3d and E3c enables us to confirm that a simple dissociation of E3 from PDC, namely, change of E3c to E3d without any interactions, is unlikely upon heat treatment of PDC.

A heat treatment of E3d induced the blue shift of the fluorescence of ANS added and raised the fluorescence intensity of ANS notably. The increase in ANS fluorescence was slightly dependent on the protein.
concentration of E3d. Gel filtration analyses showed that the inactive aggregates are responsible for increase in ANS fluorescence. The aggregation reaction was also reflected in an increase in light scattering of E3d solution. It was reported that the pig heart E3 does not bind ANS, but its FAD-free apoenzyme binds ANS with a remarkable increase in the fluorescence. The binding of ANS occurs at the same site as that of FAD and requires the conformation of the apoenzyme. Since we neither prepared the apoenzyme of E3d nor examined the ANS binding to the apoenzyme directly, we so far have no information on such a specific binding of ANS except the binding to hydrophobic site(s). Since a Trp residue and an FAD molecule are in an E3 polypeptide, these two are useful as intrinsic probes for spectroscopic inspection on temperature-induced changes in E3d.

On the other hand, the understanding of the relationship between changes in Trp fluorescence of PDC and changes in E3c was difficult, because all component polypeptides of PDC have at least one Trp residue each and because drastic changes in the other component enzymes, especially E1, precede. A heat treatment of E3d raised the intensities of Trp and FAD fluorescence; the increment as a function of treatment temperature was well correlated to each other. Both changes might proceed without E3d-E3d interaction; the values of corresponding rate constants were similar and independent of protein concentration of E3d. Results from gel filtration analysis indicated that, after heat treatment, the fluorescence ascribed to the Trp residue is emitted from the aggregate and that the fluorescence ascribed to FAD is emitted from a molecular species smaller than E3d. One of the possible mechanisms of increasing fluorescence is the destruction of the charge transfer complex between FAD and the Trp residue; it is detected in enzymes such as Azotobacter vinelandii E3. We so far have no data to confirm this mechanism, although it is likely that the changes in the Trp residue are closely related to those in the FAD molecule. The very low intensity of the FAD fluorescence of E3d, which is less than the fluorescence intensity of free FAD, hinders further analysis. We have a question whether the molecular species smaller than E3d is an FAD molecule completely dissociated from E3d, considering the following results. FAD in the solution of E3d inactivated by heat treatment was incompletely dialyzed against a buffer. The fluorescence intensity of FAD raised by heat treatment of E3d approached that of free FAD, but the intensity was still lower than that of a free FAD. The heat treatment reduced the fluorescence anisotropy of FAD in E3d, but it was slightly larger than that of free FAD (Nishimoto et al., unpublished results). Recent measurements of the time-resolved fluorescence of FAD in E3d during incubation at high temperatures have given the following intimation; a heat treatment makes the movement of the isoalloxazine ring of FAD larger, but an FAD molecule is still surrounded by steric hindrances probably within a polypeptide (Yamashita et al., unpublished results). Results from gel filtration and dialysis, however, suggest that the inactivation of E3d is accompanied by the release of FAD molecules even if it is incompletely done. The gel filtration and dialysis were done at 30°C and 4°C, respectively, after heat treatment; upon gel filtration, the temperature of an E3d solution was elevated, kept constant, reduced, and elevated again. The overall inactivation reaction of E3d is suggested to be irreversible, but if the reaction involves some reversible steps (equilibrium steps), the equilibrium might shift dependently on changes in temperature. One of other explanations is that many molecules of FAD dissociated from E3d are trapped in a small amount of inactive E3d polypeptide. We need further experiments to clarify the destiny of FAD molecules. The reconstitution of the Azotobacter vinelandii and Pseudomonas fluorescens E3's were reported to involve at least two steps: the first step is the binding of FAD to a monomeric apoenzyme, and the second is the dimerization that is essential for E3 activity. Although we have no data on an apoenzyme as described above, by measuring fluorescence anisotropy, we found that the B. stearothermophilus E3d (holo-E3) is dissociated into monomers during incubation at 65°C (Yamashita et al., unpublished results). Information on the relationship between structure and function of E3, especially the information on that of an E3 from Gram-negative bacterium, are relatively abundant. However, the documentation on thermally induced changes in E3 are quite limited. The thermoinactivation reaction of the A. vinelandii holo-E3 was reported to be biphasic; initially a rapid phase and a successive first-order kinetics phase. We could not detect such a biphasic inactivation. It was also reported that the thermostability of the A. vinelandii holo-E3 changes drastically depending on its redox state, and that the thermal inactivation of (reduced) holo-E3 results in the irreversible dissociation of FAD and the formation of monomeric apoenzyme. These results are quite interesting, but none of our studies was done under reduced conditions. Many problems of the thermal disintegration mechanisms of E3d and E3c remain so far unsolved. Based on results in this report, however, it was suggested that the microscopic changes induced in E3d by heat treatment are simpler than those in E3c. The incubation at high temperatures might cause an irreversible consecutive reaction of E3d; loss in activity, dissociation of subunit, release of FAD, and aggregation. In conclusion, the E3 enzyme is stabilized by being in the B. stearothermophilus pyruvate dehydrogenase complex.
Acknowledgments

We would like to thank Dr. Nakajima, Dr. Nagata, and Dr. Kondo (Unitika Ltd.) for providing frozen cell paste of *B. steaerothermophilus* and useful information on "Diaphorase II". We also would like to acknowledge the continuing guidance and encouragement of Dr. Ueki (JASRI) and Dr. Fujisawa (RIKEN).

References


