Rapid Communication J. Biochem. 123, 564-567 (1998)

Guanidine Hydrochloride-Induced Changes of the E2 Inner Core of the *Bacillus stearothermophilus* Pyruvate Dehydrogenase Complex[1]

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Received for publication, October 31, 1997

The limited proteolysis of the *Bacillus stearothermophilus* pyruvate dehydrogenase complex by V8 protease yields its core structure solely composed of lipoate acetyltransferase (E2) fragments. The changes in the core with guanidine hydrochloride (GdnHCl) were biphasic: below 0.8 M (first) and above 1.0 M (second) GdnHCl. The changes in the first phase were slight but significant: decreases in ellipticity and light scattering, and an increase in E2 activity. Insignificant changes in the molecular shape and size of the core were detected on fluorescence spectroscopy, ultracentrifugation, gel filtration, and electron microscopy. On the other hand, the changes in the second phase were drastic; the core was disassembled and denatured.

Key words: *Bacillus stearothermophilus*, denaturation, guanidine hydrochloride, multienzyme complex, pyruvate dehydrogenase.

A variety of enzyme assemblies are present in a living cell. Even some enzymes isolated *in vitro* are considered to be assembled *in vivo* (*1*). We have been interested in the stabilizing mechanism of a large-scale assembly of polypeptides. Information available for elucidating the mechanism is limited, compared with the elegant theories for a monomeric enzyme based on the well-documented relationship between structure and function. Discussion of the stability of an oligomeric enzyme is not straightforward, because numerous reactions involved in its structural changes should be taken into account; *e.g.* inactivation/reactivation, dissociation/reassociation, unfolding/refolding, reconstitution, and aggregation (*2*). It is difficult to distinguish clearly between two of these reactions, and an increase in the number of components might complicate the discussion further. However, we insufficiently understand even events during and after the disintegration of a large complex of proteins. The pyruvate dehydrogenase complex (PDC) from *Bacillus stearothermophilus* is a multienzyme complex comprising pyruvate decarboxylase [EC 1.2.4.1] (E1), lipoate acetyltransferase [EC 2.3.1.12] (E2), and dihydrolipoamide dehydrogenase [EC 1.6.4.3] (E3); these components facilitate the acetyl transfer from pyruvate to CoA (*3, 4*). Sixty E2 polypeptides are non-covalently assembled to form the core structure of PDC (E2 core), and many E1 and E3 polypeptides are non-covalently attached to the E2 core; the sedimentation coefficient and molecular size of PDC have been estimated to be 74S and 7-10 MDa, respectively (*5, 6*). The interactions between component polypeptides isolated from PDC or overexpressed in *Escherichia coli* have been extensively investigated (*7-11*), but only limited data on changes in the entire structure of PDC have been reported. We have examined temperature-, KI-, and guanidine hydrochloride (GdnHCl)-induced changes in PDC, and one of our speculations is that such disturbances at low levels reduce the molecular size of PDC without the dissociation of its components (*12-15*). It has been reported that limited proteolysis of PDC releases the lipoyl and E1-E3 binding domains from an E2 polypeptide, yielding an assembly composed of 60 identical E2 fragments: the E2 inner core (E2ic) (*16-20*). Although the structure of E2ic is fairly simple compared to that of PDC, the stability of E2ic might contribute to that of PDC. The results of studies on the disintegration of E2ic, therefore, are expected to be informative. With this view, this study was undertaken to examine the changes of E2ic in the presence of GdnHCl (*21*).

Unless otherwise noted, 25 mM potassium phosphate buffer (pH 7) containing 0.1 M NaCl was used and is referred to as buffer A. PDC was purified from *B. stearothermophilus* NCA 1503 (*6*). E2ic was prepared by the method of Packman *et al.* with minor modifications (*18*). A PDC solution (2 ml, 6.58 mg/ml) was dialyzed at 4°C for 12 h against 12 mM sodium phosphate buffer (pH 7) containing 0.7 mM EDTA, mixed with 0.1 ml of the phosphate buffer containing 0.1 mg of *Staphylococcus aureus* V8 protease (Sigma Chemical, Tokyo), and then incubated at 30°C for 12 h. The resulting precipitate was collected by centrifugation, washed twice with 0.4 ml of the phosphate buffer, dissolved in buffer A, and then centrifuged. The

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

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Abbreviations: E2, lipoate acetyltransferase; E2ic, E2 inner core; GdnHCl, guanidine hydrochloride; PDC, pyruvate dehydrogenase complex.

resulting supernatant contained 2.73 mg of E2ic. On SDS-PAGE, the apparent molecular size of the component polypeptide of E2ic was confirmed to be 28 kDa, as previously reported (*18*); trace amounts of E1 and E3 were detected in the preparation. The gel filtration chromatography of E2ic resulted in its elution as a single protein peak (Fig. 3A), and an open pentagonal dodecahedron of 23 nm in diameter was detected on electron microscopy (Fig. 3B); the geometrical shape was similar to that of the 33S-core prepared from PDC by tryptic digestion (*16*). The analytical ultracentrifugation of E2ic was performed at 25,000 rpm and 20°C by the sedimentation velocity method with a Beckman XL-A analytical ultracentrifuge and an An-60 Ti rotor. The boundary profiles in 1.2-cm double sector cells were monitored at 240 and 280 nm during ultracentrifugation and subsequently analyzed with the Beckman XLAVEL program. The sedimentation coefficient value of E2ic was independent of the protein concentration between 0.1 and 1.8 mg/ml, and $S^{\circ}_{20,w}$ was evaluated to be 30S. It has been reported that E2ic (5.6 mg/ml) is sedimented as a predominant peak at 27S, and that a minor peak at 40S is also detected for some preparations (*18*). We did not detect such a minor peak and thus used this preparation as intact E2ic.

Preliminarily, we monitored the Trp-fluorescence and light-scattering intensities of E2ic during incubation at 30°C for several hours. Within 5 h, both the intensities decreased notably in the presence of a high concentration of GdnHCl. Such changes were detected even in the absence of GdnHCl, although the decreases were far less than those in the presence of GdnHCl. Upon measurement at 30°C after incubation at 4°C, such spontaneous changes were not detected, but it took several days until the GdnHCl-induced spectroscopic changes settled down. The spontaneous changes remain unexplained. The incubation, therefore, was performed at 4°C for 120 h in the presence or absence of GdnHCl, and subsequent measurements were performed at 20-30°C; little changes were detected during the measurements. As described below, the spectroscopic changes were drastic in the presence of GdnHCl at around 1.0 M, while the changes were slight below this concentration. Therefore, we divided the concentration range of GdnHCl tentatively into the G-I and G-II ranges, *i.e.* between 0.1 and 0.8 M, and 1.1 and 2.0 M, respectively. As shown in Fig. 1, the far-UV CD spectra in the G-I and G-II ranges were steeper and shallower, respectively, than those of intact E2ic; the ellipticity at 222 nm decreased, but increased in G-I and G-II, respectively. As shown in Fig. 2A, upon excitation at 295 nm, the emission maximum of E2ic was 333 nm in the absence of GdnHCl and in the G-I range, but shifted to 350 nm in G-II; the fluorescence spectrum in the presence of 2 M GdnHCl was similar to that of *N*-acetyl-L-tryptophanamide. The fluorescence intensity at 330 nm increased slightly in G-I, but notably decreased in G-II. Trp-21 and Trp-355 are in an E2 polypeptide, and only the latter residue remains putatively in E2ic after limited proteolysis by V8 protease (*19, 20*). It was, therefore, speculated that Trp-355 is fully exposed to the solvent in G-II. The apparent midpoints of the conformational transition judged from changes in CD and fluorescence spectra were 1.4 and 1.0 M GdnHCl, respectively. These results suggested that an E2 polypeptide is unfolded in G-II and that the structure in the vicinity of Trp is relatively fragile. Light scattering is a sensitive and convenient measure reflecting changes in the entire molecular size of E2ic, because E2ic is much larger than a common oligomeric protein. As shown in Fig. 2B, the light scattering intensity decreased in both G-I and G-II, and the decrease at around 1.0 M GdnHCl was drastic. Upon incubation with 1.5 M GdnHCl, the core structure could not be visualized on electron microscopy, and the sedimentation coefficient in the presence of 2.0 M GdnHCl was estimated to be 3.2S. These results suggested that E2ic is dissociated in the G-II range being unfolded. The decrease in light scattering in G-I implied that the molecular size of E2ic also decreases in this range with increasing concentrations of GdnHCl. As shown in Fig. 2B, in G-I, the E2 activity increased to 130% of the original value, while E2ic was inactivated in G-II. Since we measured the activity using a GdnHCl-free substrate solution, GdnHCl is rapidly diluted 1:48 upon the assay (see the legend to Fig. 2). The increase in activity, therefore, might occur in the presence of GdnHCl or during dilution. The removal of GdnHCl by dialysis could never restore the activity lost in G-II. Recently, Allen and Perham expressed the catalytic domain (starting from Ala-173) of E2 in *E. coli*, and obtained an icosahedral 60-mer assembly of the domains (*23*). They reported that the assembly completely dissociated and inactivated in the

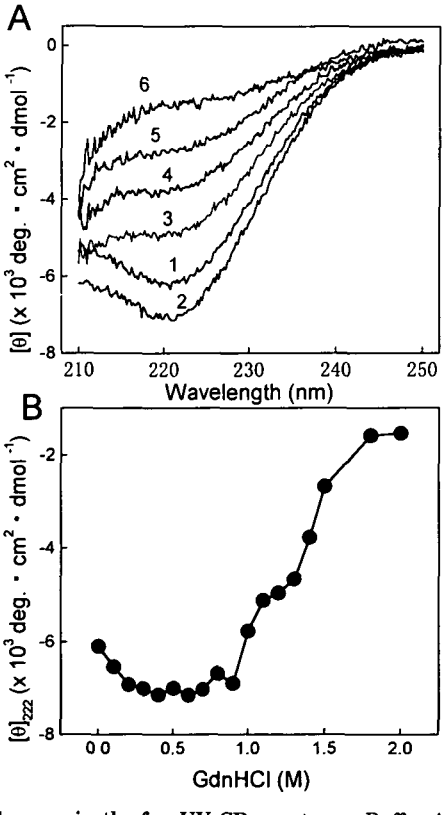

Fig. 1. **Changes in the far-UV CD spectrum.** Buffer A (0.95 ml) containing GdnHCl was mixed with 0.05 ml of 1.82 mg/ml E2ic, followed by incubation for 120 h at 4°C. The CD spectrum between 210 and 250 nm was monitored at 25°C with a Jasco J-720 spectropolarimeter and a 3-mm cell. Mean residue ellipticity was calculated based on the amino acid sequence of E2 (*19, 20*). (A) Typical CD spectra in the absence and presence of GdnHCl: 1, 0.0 M; 2, 0.5 M; 3, 1.2 M; 4, 1.4 M; 5, 1.5 M; 6, 2.0 M. (B) Mean residue ellipticity at 222 nm (●).

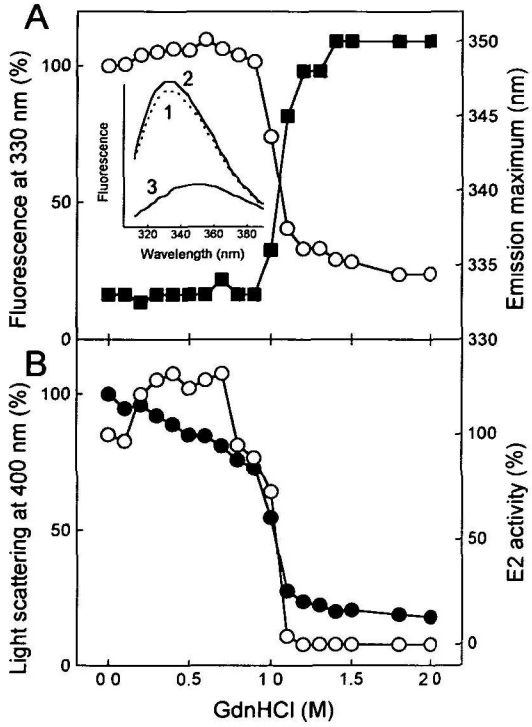

Fig. 2. **Changes in fluorescence, light scattering, and E2 activity.** Before any measurements, E2ic was incubated with GdnHCl by the method described in the legend to Fig. 1. (A) The fluorescence spectrum between 300 and 400 nm upon excitation at 295 nm was monitored at 30°C with a Hitachi 650-60 fluorescence spectrophotometer. ■: Emission maximum. The fluorescence intensity at 330 nm (○) is plotted as a percentage of that detected in the absence of GdnHCl. Inset: Typical fluorescence spectra in the absence and presence of GdnHCl: 1, 0.0 M (dotted line); 2, 0.5 M; 3, 2.0 M. The ordinate is in arbitrary units. (B) The light scattering at right angles was also monitored at 30°C with the spectrophotometer, both the excitation and emission wavelengths being 400 nm. The light scattering at 400 nm (●) is plotted as a percentage of that detected in the absence of GdnHCl. The E2 activity was assayed using dihydrolipoamide prepared by the method of Reed *et al.* (*22*). For 3 min at 30°C, 0.92 ml of 87 mM Tris-HCl buffer (pH 7.5) containing 0.54 mM dithiothreitol, 0.11 mM CoA, 5.4 mM acetyl phosphate, and 1.4 U of *Leuconostoc mesenteroides* phosphoacetyltransferase (Wako) was incubated in a quartz cell. After mixing with 20 μl of 50 mM dihydrolipoamide (ethanol) and 20 μl of an E2ic solution, the increase in absorbance at 240 nm was continuously monitored. The activity (○) is plotted as a percentage of that of intact E2ic.

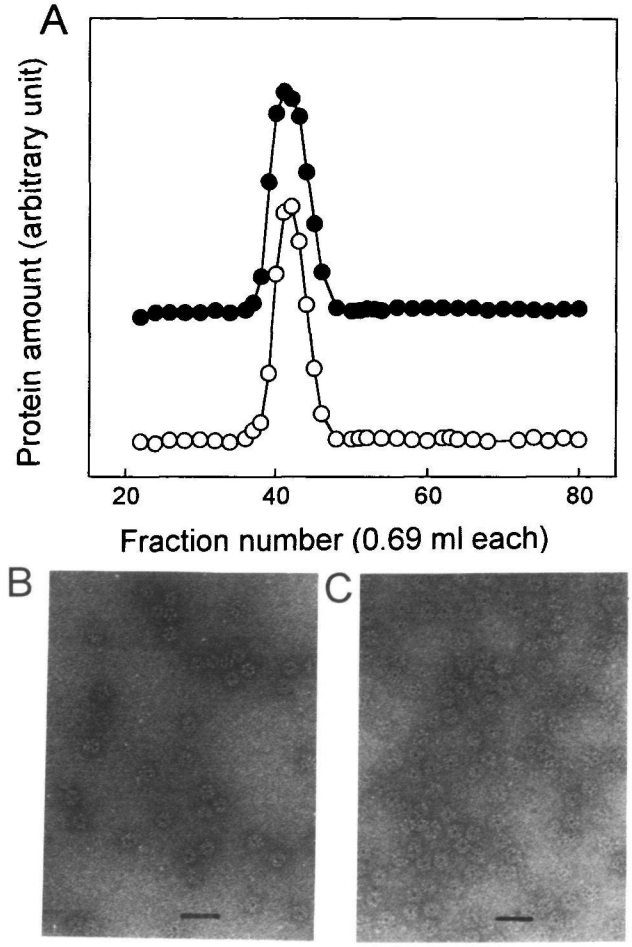

Fig. 3. **Gel filtration chromatogram and electron micrograph.** (A) Buffer A (0.6 ml) containing 1.06 mg/ml E2ic was incubated at 4°C for 120 h in the absence (○) and presence (●) of 0.5 M GdnHCl, and then chromatographed on a Sephacryl S-400HR column (1.1 × 102 cm) using buffer A and buffer A containing 0.5 M GdnHCl, respectively. Intact E2ic (B) and E2ic incubated with 0.5 M GdnHCl (C) by the method described in the legend to Fig. 1 were negatively stained with 1% sodium phosphotungstate. Electron micrographs were taken at a nominal magnification of 30,000-fold under a JEOL 2000 FX electron microscope. Bar = 50 nm.

presence of 6 M GdnHCl is reassembled and reactivated through a specially designed method of removal of GdnHCl: a gradual decrease in the GdnHCl concentration through multi-step dialysis (*23*). The E2 and E3 activities of PDC were completely abolished in the presence of GdnHCl above 2 M, and 70-90% of their original activities were restored on the removal of GdnHCl by dialysis for 48 h (Meno *et al.*, unpublished results). Although so far we have insufficient information on the reactivation of E2ic (starting from Lys-180), which is smaller than the catalytic domain described above (*19*), it was speculated that the binding of E3 to E2 facilitates the restoration of an active E2 core during the reactivation of PDC, whereas a lack of such a mechanism requires careful removal of GdnHCl for the reactivation of E2ic.

We examined the changes of E2ic in the G-I range further. E2ic treated with 0.5 M GdnHCl was submitted to gel filtration in the presence of GdnHCl, and its elution profile was compared with that of intact E2ic in the absence of GdnHCl. As shown in Fig. 3A, there was an insignificant difference between the two profiles, and neither aggregated nor dissociated polypeptides were detected. On electron microscopy, it was observed that the shape and dimensions of E2ic treated with 0.5 M GdnHCl are similar to those of intact E2ic (Fig. 3, B and C). The sedimentation coefficient value of E2ic in the presence of 0.5 M GdnHCl was 27S over the protein concentration range of 0.15 to 2.0 mg/ml. Since the sedimentation coefficient is a function of various factors, such as the partial specific volume and the frictional coefficient, critical assessment of the differences between S-values in different solvents was difficult. The S-value in 0.5 M GdnHCl, however, indicated a lack of drastic changes in molecular size. On the other hand, the concave and convex dependencies of the ellipticity and E2 activity, respectively, on the GdnHCl concentration and the de-

crease in light scattering suggested that E2ic in the G-I range undergoes a certain change in structure. Some changes in G-I were speculated, as described below. With increasing ionic strength, the molecular dimensions (probably the radius of the core) decreases, while the hydration of E2ic increases; the former change is responsible for the decrease in light scattering, but the latter compensates for the changes detected on ultracentrifugation and gel filtration. These changes increase the structural regularity of the E2 polypeptide somewhat and the E2 activity. The plausibility of this mechanism is partly based on that E2ic has a hollow structure (*25*). It has been reported that the exposure of the *Azotobacter vinelandii* E2 core, comprising 24 subunits of intact E2, to GdnHCl brings about a decrease in light scattering with two transitions at 1.0 and 1.8 M, and that the decrease is due to the two-step dissociation of the core (*24*). If E2ic is dissociated in G-I, the dissociation might be followed by instantaneous association, like that of 29 E2ic (60-mer) to 30 E2ic (58-mer); a decrease in molecular size without the appearance of free components. A decrease in the light scattering of E2ic without a great change in the Trp-fluorescence was also observed in 1-2 M NaCl or urea (data not shown). Furthermore, we found that the incubation of E2ic at temperatures above 72°C yields small amounts of inactive aggregates (A. Nakajima *et al.*, unpublished results). Therefore, we cannot exclude that trace amounts of aggregates originally in the E2ic preparation were dissociated into active E2ic on the addition of a reagent.

We are grateful to Drs. H. Nakajima and K. Nagata (Unitika Co., Ltd.) for providing the frozen *B. stearothermophilus* cell paste. We are also indebted to Dr. S. Yamashita (Kyushu University) for the fluorescence measurements.

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