Effects of Guanidine Hydrochloride on Pyruvate Dehydrogenase Complex

Meno, Kohji
Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Aso, Yoichi
Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University

Hiromasa, Yasuaki
Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Irie, Toshiro
Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

他

https://doi.org/10.5109/24462

出版情報：九州大学大学院農学研究院紀要. 47 (1), pp.77-87, 2002-10-30. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **47** (1), 77–87 (2002)

Effects of Guanidine Hydrochloride on Pyruvate Dehydrogenase Complex

Kohji MENO*, Yoichi ASO, Yasuaki HIROMASA*, Toshiro IRIE*, Masahito KITA*, and Masatsune ISHIGURO**

Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(Received June 6, 2002 and accepted July 12, 2002)

Many molecules of pyruvate decarboxylase (E1) and dihydrolipoamide dehydrogenase (E3) bind non–covalently to a sixty–meric lipoate acetyltransferase (E2), forming the *Bacillus stearothermophilus* pyruvate dehydrogenase complex. Changes in the complex induced by incubation in guanidine hydrochloride (GdnHCl) solution and those induced by the incubation followed by removal of GdnHCl were examined. Small amount of GdnHCl induced reversible changes in the complex. The removal re–associated all components to the complex and restored the enzyme activities of the complex and its components. NaCl induced similar changes, and the effect of GdnHCl was implied as a salt rather than a denaturant. On the other hand, larger amount of GdnHCl induced irreversible changes in the complex. E1, E2, and E3 were dissociated and inactivated; FAD was released from E3. GdnHCl at concentrations limited to a small range around 1.0 M induced a partial and irreversible aggregation. The removal of GdnHCl yielded an E2–E3 complex (57S), restoring most activities of E2 and E3. Loss in enzyme activity of E1 might be responsible for that of the complex.

INTRODUCTION

The *Bacillus stearothermophilus* pyruvate dehydrogenase complex (PDC) is a non–covalent assembly of nearly two hundred polypeptides (Perham, 1996; Domingo *et al.*, 1999; Izard *et al.*, 1999). The three component enzymes of PDC 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). E1, E2, and E3 are hetero–tetramer, homo–sixty–mer, and homo–dimer, respectively: $(E1\alpha)_2(E1\beta)_2$, $(E2\alpha)_{60}$, and $(E3\alpha)_2$. The E2α polypeptide has lipoyl, subunit binding, and catalytic domains (Perham, 2000). The catalytic domain is essential for the association of sixty E2α polypeptides besides the catalytic function of E2 (Zhou *et al.*, 2001). Either E1 or E3 is attached to the subunit binding domain, and a lipoyl group is covalently attached to the lipoyl domain. E1 catalyzes the decarboxylation of pyruvate and the transfer of an acetyl group from pyruvate to thiamine diphosphate. E2 catalyzes the acetyl transfer from the thiamine diphosphate to CoA, using the lipoyl group. The transfer results in the reduction of the lipoyl group to a dihydrolipoyl group. E3 catalyzes the re–oxidation of the dihydrolipoyl group, using reduction systems from FAD to $FADH_2$ and from NAD^+ to $NADH_2$. PDC thus facilitates the synthesis of an acetyl CoA; we refer to the capability of catalyzing this overall reaction

* Laboratory of Protein Chemistry and Engineering, Department of Genetic Resources Technology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

** Corresponding author (E–mail: yaso@agr.kyushu–u.ac.jp)

as PDC activity. We have been concerned with disintegration mechanism of a large-scale polypeptide complex. PDC is a relatively well documented complex, and its assembly structure is simpler than that of a mammalian PDC (Roche *et al.*, 1996). Only limited information on disintegration mechanism of PDC is, however, available because of little knowledge of multiple interactions in PDC. In practice, the disintegration of PDC involves multiphasic reactions rather than a simple dissociation into components (Hiromasa *et al.*, 1993; Hiromasa *et al.*, 1994; Hiromasa *et al.*, 1997; Aso *et al.*, 1998). We therefore have considered that the accumulation of basic data is still required for understanding of the mechanism. The present study has been undertaken to know the outline of guanidine-hydrochloride induced changes in PDC.

MATERIALS AND METHODS

Chemicals and enzyme

All chemicals 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, 0.15 mM phenylmethanesulfonyl fluoride, and 0.02% NaN_3: standard buffer. The amount of guanidine hydrochloride (GdnHCl) was confirmed by measuring refractive index (Pace *et al.*, 1989). FAD was fluorimetrically measured by the method previously reported (Hiromasa *et al.*, 1997). Protein amount was measured with a BioRad protein assay kit using bovine serum albumin as standard protein. PDC was purified from *B. stearothermophilus* (NCA 1503) according to the method reported previously (Hiromasa *et al.*, 1995) and stored as precipitates in the presence of 60% saturation of $(NH_4)_2SO_4$ at 4 °C before use. E1, E2, E3, and PDC activities were measured at 30 °C by the methods reported previously (Hiromasa *et al*, 1993; Hiromasa *et al.*, 1995).

Incubation with and removal of reagent

A standard buffer containing 0.11 mg/ml PDC and various amounts of GdnHCl or NaCl was incubated at 4 °C for 24 h. Changes in PDC were measured immediately or after removal of the reagent; in the former case, measurements were done in the presence of the same amount of the reagent as that upon incubation, while, in the latter case, measurements were done in the absence of the reagent. The removal of the reagent was done by dialysis against standard buffer at 4 °C for 48 h.

Spectroscopic measurements

Spectroscopic measurements were done at 30 °C with a Hitachi 850 fluorescence spectrophotometer. Fluorescence spectra ascribed to 8-anilinonaphthalene-1-sulfonate (ANS) and FAD were measured upon excitation at 350 nm and 460 nm, respectively. For the measurement of ANS fluorescence, a test solution was titrated with 0.024 mM ANS until its fluorescence intensity reached a plateau. The intensity of light scattering was measured at a right angle by setting both the excitation and emission wavelengths at 540 nm.

Ultracentrifugation and gel filtration

Ultracentrifugation was done at 20,000 rpm with a Beckman XL-A analytical ultracen-

trifuge and an An–60 Ti rotor by the sedimentation velocity method previously reported (Hiromasa *et al.*, 1998a). Gel filtration was done on a Sepharose CL–2B column (0.8× 103 cm) at 4 °C with standard buffer in the presence or absence of GdnHCl.

RESULTS

Changes in enzyme activities

In the presence of GdnHCl, PDC was incubated for 24 h, and its enzyme activities were measured. PDC and E1 activities decreased monotonously with increasing amount of GdnHCl (Fig. 1A & 1B). Both the activities were less than 10% of their original values in 0.3 M GdnHCl. On the other hand, E2 and E3 activities were fully retained or slightly raised in the presence of GdnHCl at concentrations less than 0.1 M and 0.3 M, respectively (Fig. 1C & 1D). Above these concentrations, E2 and E3 activities decreased with increasing amount of GdnHCl. E2 and E3 activities were less than 10% in 0.5 M and 1.5 M GdnHCl, respectively. Furthermore, effects of removal of GdnHCl were examined. After PDC had been incubated in the presence of GdnHCl, GdnHCl was removed by dialysis. The removal of relatively small amounts of GdnHCl raised PDC activity at the same or higher level than original activity (Fig. 1A). However, the activity lost in the presence of

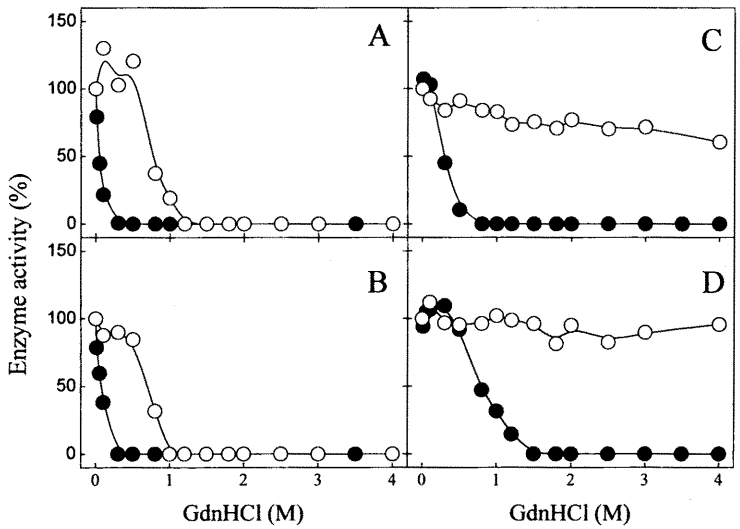

Fig. 1. GdnHCl-induced changes in enzyme activities. After incubation of PDC in the presence of GdnHCl, PDC (A), E1 (B), E2 (C), and E3 (D) activities were measured (●). Separately, after incubation of PDC in the presence of GdnHCl and removal of GdnHCl by dialysis, PDC (A), E1 (B), E2 (C), and E3 (D) activities were measured in the absence of GdnHCl (○). Control experiments were done in the same manner as described above except for the absence of GdnHCl. Data are plotted as relative values by taking the corresponding data obtained from control experiment as 100%.

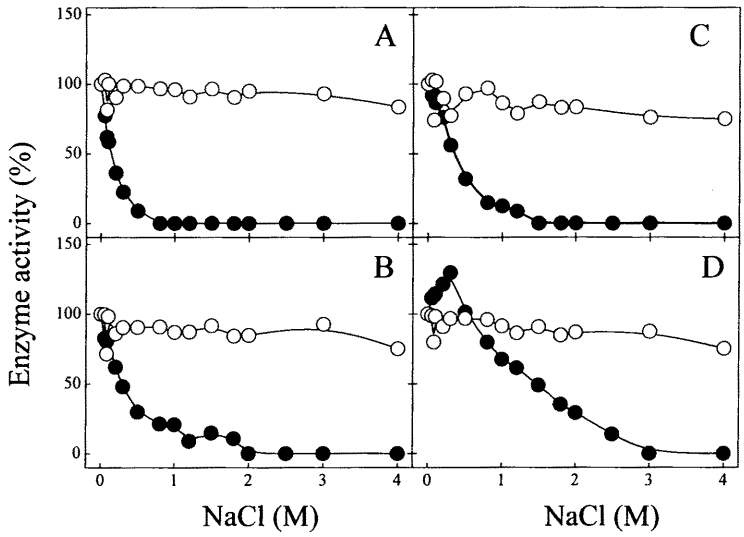

Fig. 2. NaCl-induced changes in enzyme activities. After incubation of PDC in the presence of NaCl, PDC (A), E1 (B), E2 (C), and E3 (D) activities were measured (●). Separately, after incubation of PDC in the presence of NaCl and removal of NaCl by dialysis, PDC (A), E1 (B), E2 (C), and E3 (D) activities were measured in the absence of NaCl (○). Control experiments were done in the same manner as described above except for the absence of NaCl, and data are plotted as relative values by taking the corresponding data obtained from control experiment as 100%.

GdnHCl at concentrations above 1.0 M was never recovered. Changes in E1 activity were similar to those of PDC activity, although E1 activity never became higher than its original activity (Fig. 1B). The removal restored the large portions of E2 and E3 activities lost (Fig. 1C & 1D). The recoveries of these activities were relatively high; 75% of E2 and E3 activities lost in 6 M GdnHCl were recovered.

Effects of NaCl were examined by similar methods. Changes in PDC, E1, E2, and E3 activities were similar to corresponding changes in GdnHCl, although the activities decreased, more gently depending on amount of NaCl (Fig. 2). PDC, E1, E2, and E3 activities were less than 10% of their original values in 0.5 M, 1.2 M, 1.2 M, and 3.0 M NaCl, respectively. The removal of NaCl restored 75–80% of E1, E2, E3, and PDC activities lost in 4 M NaCl (Fig. 2).

Spectroscopic changes

After incubation of PDC for 24 h in the presence of GdnHCl, light scattering, fluorescence of extrinsic ANS, and fluorescence of intrinsic FAD were measured. GdnHCl at concentrations around 1.0 M raised the intensity of light scattering notably, and GdnHCl above this concentration reduced the intensity to 5–10% of value of intact PDC (Fig. 3A).

The removal of GdnHCl made the intensity of light scattering almost the same level as that of intact PDC (Fig. 3A). Changes of the intensity of ANS fluorescence peaked also in 1 M GdnHCl (Fig. 3B). The removal of GdnHCl reduced the intensity, but the intensity by the removal of GdnHCl at concentrations over 1 M reached a plateau; the intensity was higher than that of intact PDC (Fig. 3B). Similar results were obtained from the following experiment; without dialysis, a PDC solution containing GdnHCl was rapidly diluted ten times just before the measurement of ANS fluorescence. Changes in fluorescence intensity showed a similar tendency to those from dialysis, but the intensity at plateau region was 2.5 times as high as that yielded by dialysis. Changes in FAD fluorescence were different. GdnHCl induced a red shift of fluorescence spectrum of FAD; the emission maximum was shifted from 512 nm to 516 nm and to 527 nm in the presence of 0.5 M and 2.0–5.7 M, respectively. The intensity of the FAD fluorescence increased in GdnHCl at concentrations above 1 M (Fig. 3C); the intensity at 4 M was five times as high as that in

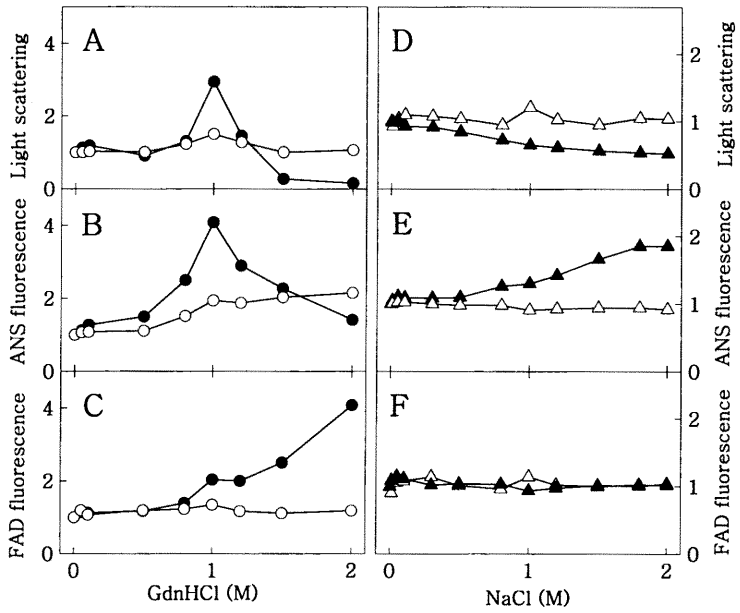

Fig. 3. Spectroscopic changes. Panel A, B, and C: After incubation of PDC in the presence of GdnHCl, intensities of light scattering at 540 nm (A), ANS fluorescence at 480 nm (B), and FAD fluorescence at 510 nm (C) were measured (●). Separately, after incubation of PDC in GdnHCl and removal of GdnHCl by dialysis, these were also measured in the absence of GdnHCl (○). Panel D, E, and F: Except for using NaCl instead of GdnHCl, experimental conditions were same as those for (A)–(C). Intensities of light scattering (D), ANS fluorescence (E), and FAD fluorescence (F) were measured in the presence (▲) or absence (△) of NaCl. All the intensities are plotted as relative values by taking the corresponding intensity of PDC in the absence of GdnHCl and NaCl as one.

the absence of GdnHCl. The removal of GdnHCl restored the intensity of the fluorescence (Fig. 3C) and the emission maximum.

Spectroscopic changes induced with NaCl were less than those with GdnHCl. With increasing amount of NaCl, the intensity of light scattering decreased (Fig. 3D), but the intensity of ANS fluorescence increased (Fig. 3E). The removal of NaCl canceled these changes almost completely. Both the incubation in the presence of NaCl and its removal affected the intensity of FAD fluorescence insignificantly (Fig. 3F); these were also ineffective on the emission maximum of FAD fluorescence.

Changes in molecular size

Intact PDC was eluted from a gel filtration column as a single peak of protein at fraction No. 52 (Fig. 4A–a). The peak shifted to No. 54 in 0.2 M GdnHCl (Fig. 4A–b). In 0.5 M GdnHCl, the peak shifted to No. 55, and a shoulder appeared at No. 72 (Fig. 4A–c). Three protein peaks were detected at No. 30, No. 58, and No. 72, respectively, in 1.0 M GdnHCl (Fig. 4A–d). The first peak was at the exclusion limit of the column and emitted ANS fluorescence greatly; emission from other molecular species including intact PDC was faint (Fig. 4B). In 1.5 M GdnHCl, the great part of protein was eluted around No. 74 (Fig. 4A–e). Analyses by SDS polyacrylamide gel electrophoresis (SDS–PAGE) gave the following results, although we failed to discriminate between E1α and E1β polypeptide bands on a gel; the peak at No. 30 was rich in E1 polypeptide; the peak at No. 58 was

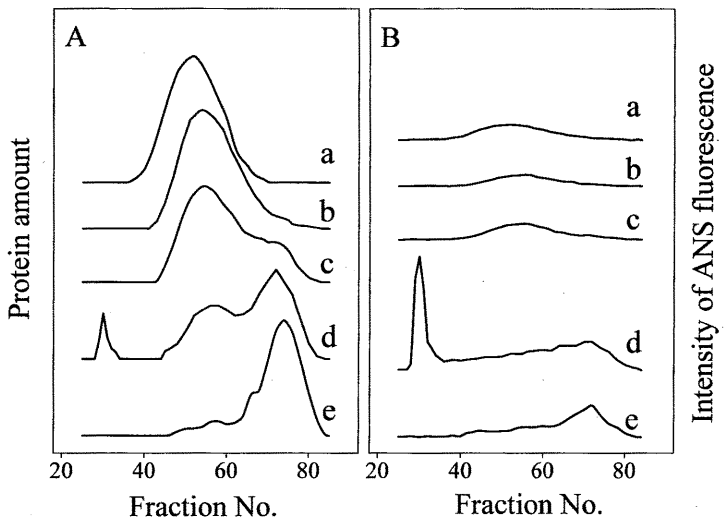

Fig. 4. Gel filtration chromatography in the presence of GdnHCl. PDC (0.6 ml) incubated in 0.0 M (control) (a), 0.2 M (b), 0.5 M (c), 1.0 M (d), and 1.5 M (e) GdnHCl were separately filtered on a Sepharose CL–2B column with standard buffer containing same amount of GdnHCl. Volume of a fraction was 0.65 ml. Protein amount (A) and intensity of ANS fluorescence at 480 nm (B) are plotted in arbitrary units.

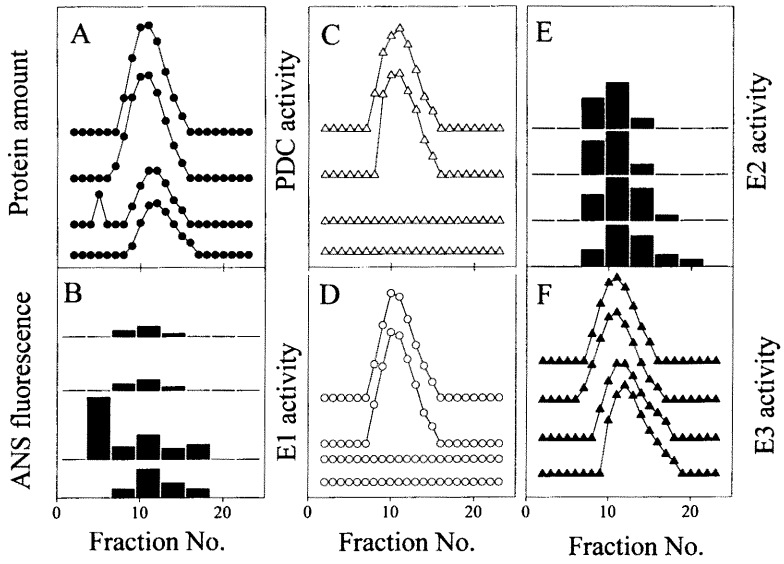

Fig. 5. Gel filtration chromatography after removal of GdnHCl. PDC was incubated in the presence of 0.0 M (control), 0.5 M, 1.0 M, and 1.5 M GdnHCl: chromatograms from top to bottom in each panel. GdnHCl was removed by dialysis, and each 0.7 ml-solution was filtered on a Sepharose CL-2B column with standard buffer. Volume of a fraction was 3.3 ml. Protein amount (A), intensity of ANS fluorescence at 480 nm (B), PDC activity (C), E1 activity (D), E2 activity (E), and E3 activity (F) are plotted in arbitrary units. ANS fluorescence (B) and E2 activity (E) are expressed as bar graphs; these were measured for corresponding fractions combined.

almost exclusively composed of E2α polypeptide; the peak at No. 74 was rich in E1 and E3α polypeptides (data not shown).

After removal of GdnHCl by dialysis, the gel filtration of PDC was done in the absence of GdnHCl. Resulting fractions were examined as to distributions of protein, intensity of ANS fluorescence, and enzyme activities (Fig. 5). Incubation with 0.5 M GdnHCl affected the elution profile of intact PDC insignificantly. E1 and PDC activities lost in the presence of 1.0–1.5 M GdnHCl were never restored, while E2 and E3 activities were recovered, showing slightly larger elution volumes (Fig. 5E & 5F). The elution profile of FAD coincided with that of E3 activity (data not shown). Aggregate in 1.0 M GdnHCl remained after the removal of GdnHCl and emitted ANS fluorescence greatly (Fig. 5B). Five fractions around a protein peak were separately combined, concentrated, and subjected to a sedimentation velocity analysis. Single molecular species was detected in a peak after removal of 0.5 M GdnHCl, and its sedimentation coefficient ($S^{\circ}_{20,w}$) was the same as that of intact PDC: 75 S. Molecular species after removal of 1.0 M or 1.5 M GdnHCl was also single, and both their coefficients were evaluated 57 S. SDS–PAGE analysis indicated

that the polypeptide composition of a 75 S–species is quite similar to that of an intact PDC, while a 57 S–species contains larger amounts of E2 and E3 than intact PDC (data not shown).

DISCUSSION

PDC activity is one of the most sensitive probes to monitor changes in PDC, because the activity is solely performed by assemblage of active component enzymes. E1, E2, and E3 activities are independently measured of each other with convenient substrates and may reflect changes in their molecular states. However, information on changes in activities of components during the disintegration of PDC is limited. Witzmann and Bisswanger reported that the salts at concentrations less than 50 mM are ineffective on PDC activity of the *B. stearothermophilus* PDC, although they showed no data about changes above the concentration (Witzmann and Bisswanger, 1998). They also reported the inhibitory effects of NaCl and KCl on PDC activity of the *Thermus flavus* PDC: 30–40% of its original activity in 0.5 M salt (Witzmann and Bisswanger, 1998). The bovine PDC loses its PDC activity in 0.2 M GdnHCl (West *et al.*, 1995). A rapid decrease in GdnHCl concentration by dilution was reported to restore PDC activity of the bovine PDC (West *et al.*, 1995). These results implied that GdnHCl–induced changes in PDC activity are similar to each other, being independent of source of PDC. Based on changes in PDC activity, it was suggested that the effects of NaCl on PDC and those of GdnHCl at relatively low concentrations are reversible and that those of GdnHCl at higher concentrations are irreversible. E2 and E3 activities were highly recovered even from 6 M GdnHCl, whereas E1 was the most labile component. Changes in E1 activity were well correlated to those in PDC activity. Stability of E1 was, therefore, suggested greatly responsible for that of PDC. Such a correlation is also observed when PDC is inactivated thermally (Hiromasa *et al.*, 1994) or with potassium iodide (Aso *et al.*, 1998).

E3 activity increased in the presence of a little amount of salt; it was notable in NaCl. The activity of an isolated E3 is also 1.5–1.8 times that of original value in GdnHCl, NaCl, or KCl (Hiromasa *et al.*, 1998b). FAD is a cofactor exclusively bound to E3. The molar fluorescence intensities of FAD of E3 in PDC, FAD in isolated E3, and free FAD were measured in the ratios 1.0:0.3:4.0 (Hiromasa *et al.*, unpublished results). In the presence of GdnHCl, therefore, release of FAD from E3 might cause an increase in FAD fluorescence. The release was suggested reversible, because the removal of GdnHCl restored the spectrum of FAD fluorescence besides E3 activity. Upon GdnHCl–induced denaturation, isolated E3 also releases FAD (Hiromasa *et al.*, 1998b). It was therefore suggested that the increase in E3 activity and the release of FAD are independent of changes in other components. Results from light scattering measurement and gel filtration indicated the exfoliation of E1 and E3 components from E2. In 0.2 M GdnHCl, the bovine PDC release E1 and E3 completely (West *et al.*, 1995). Since the structure of the bovine PDC containing extra components is more complicated than that of the *B. stearothermophilus* PDC (De Marcucci *et al.*, 1995; McCartney *et al.*, 1997), we so far have insufficient knowledge of dissociation mechanism of E1 and E3.

Functional and 60–meric E2 is prepared by overexpression of E2 gene in *Escherihia coli* (Lessard *et al.*, 1998). E2 inner core (E2ic) is furthermore an active 60–mer smaller

than E2; E2ic has neither the binding domain nor the lipoyl domain (Packman *et al.*, 1984). Since E2 forms the core of PDC, studies on E2 and E2ic have separately been done from PDC. Except for the notable increase in E3 activity, changes in E2 activity were similar to those of E3 activity. We previously examined the effects of GdnHCl on E2ic (Hiromasa *et al.*, 1998a; Aso *et al.*, 2001). E2 activities of PDC and E2ic decreased, similarly depending on GdnHCl concentration. On the other hand, the effects of removal of GdnHCl were different from each other. E2ic lost almost all the activity irreversibly by removal of 1.2–2.0 M GdnHCl, although nearly 90% of original activity lost in 3.0–4.5 M GdnHCl is recovered (Aso *et al.*, 2001). Recently we examined the effects of GdnHCl on the activity of E2 prepared by overexpression of its gene in *E. coli* and found that the activity recovery of the isolated E2 is similar to that of PDC (Kita *et al.*, unpublished results). The truncated two domains and/or interaction of other components on the binding domain might therefore contribute to the inactivation of E2. The unfolding of isolated E2 proceeds without any intermediates; only monomer is in 2.0 M GdnHCl (Lessard *et al.*, 1998). On the other hand, the 24–meric E2 from *Azotobacter vinelandii* is disintegrated with two–step dissociation (Hanemaaijer *et al.*, 1989). We reported that changes in light scattering of E2ic is biphasic in GdnHCl, although we could never detect any intermediates (Hiromasa *et al.*, 1998a). In the present study on PDC, we failed to detect any intermediates of E2, because it was difficult to monitor dissociation of E2, discriminating from the dissociation of E1 and/or E3 from E2.

A small amount of aggregate was detected in a quite narrow concentration range of GdnHCl. The aggregate reaction was irreversible, and its product might be a byproduct rather than a final product. An increase in intensity of ANS fluorescence is generally recognized to reflect increase in hydrophobic region. The aggregation of E2ic occurs with insignificant changes in ANS fluorescence (Aso *et al.*, 2001), whereas this study revealed that the aggregate from PDC is mainly responsible for notable increase in emission of ANS fluorescence. E2ic comprising homo–polypeptides is different from E2 in PDC. The dissociation of E1 and E3 from E2 might extend hydrophobic region. Based on results from gel filtration and ultracentrifugation studies, incubation of PDC in high concentration of GdnHCl and successive removal of GdnHCl was suggested to yield an E2–E3 complex. Sixty molecules of E1 or those of E3 was reported capable of spontaneous association with an E2 (Lessard *et al.*, 1998; Domingo *et al.*, 1999). It was speculated that, upon the formation of E2–E3 complex during removal of GdnHCl, lack of E3 molecules might help binding of ANS molecules to some sites originally occupied by E1.

Although there are several reports describing the effects of denaturant on PDC, some studies have focused on the denaturation of its isolated components rather than PDC itself, others have focused on drastic changes with high concentration of denaturant to perform the reconstitution of the complex: for example, complete dissociation and incomplete reconstitution of PDC (Jaenicke and Perham, 1982). Properties of isolated component enzyme are not necessarily same as those of enzyme in a complex (Hiromasa *et al.*, 2000). Further studies on disintegration mechanism of complex and its components under mild conditions would help to understand structural properties of a gigantic protein assembly.

ACKNOWLEDGEMENTS

We would like to thank Dr. Kondo (Unitika Ltd.) for providing frozen cell paste of *B. stearothermophilus*.

REFERENCES

Allen, M. D. and R. N. Perham 1997 The catalytic domain of dihydrolipoyl acetyltransferase from the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus*. Expression, purification and reversible denaturation. *EFEBS Lett.*, **413**: 339–343

Aso, Y., Y. Hiromasa, Y. Aikawa, K. Meno and M. Ishiguro 1998 Potassium iodide–induced changes in pyruvate dehydrogenase complex from *Bacillus stearothermophilus*. *Biosci. Biotechnol. Biochem.* **62**: 108–116

Aso, Y., K. Meno, T. Fujisawa and M. Ishiguro 1999 Small–angle X–ray scattering studies on aggregation of pyruvate dehydrogenase complex. *Protein Sci.*, Suppl. 2, **8**: 61

Aso, Y., A. Nakajima, K. Meno and M. Ishiguro 2001 Thermally induced changes of lipoate acetyltransferase inner core isolated from the *Bacillus stearothermophilus* pyruvate dehydrogenase complex. *Biosci. Biotechnol. Biochem.*, **65**: 698–701

De Marcucci, O. L., M. S. DeBuysere and M. S. Olson 1995 Dissociation and reassembly of the dihydrolipoyl transacetylase component of the bovine heart pyruvate dehydrogenase complex. *Arch. Biochem. Biophys*, **323**: 169–176

Domingo, G. J., H. J. Chauhan, I. A. D. Lessard, C. Fuller and R. N. Perham 1999 Self–assembly and catalytic activity of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. *Eur. J. Biochem.*, **266**: 1136–1146

Hanemaaijer, R., A. H. Westphal, T. Van Der Heiden, A. de Kok and C. Veeger 1989 The quaternary structure of the dihydrolipoyl transacetylase component of the pyruvate dehydrogenase complex from *Azotobacter vinelandii*. *Eur. J. Biochem.*, **179**: 287–292

Hiromasa, Y., Y. Aso and S. Yamashita 1993 Purification of the pyruvate dehydrogenase complex from an extreme thermophile, *Bacillus caldolyticus*, and its thermal stability. *Biosci. Biotechnol. Biochem.* **57**: 1062–1066

Hiromasa, Y., Y. Aso and S. Yamashita 1994 Thermal disassembly of pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. *Biosci. Biotechnol. Biochem.* **62**: 1904–1905

Hiromasa, Y., Y. Aso, S. Yamashita and Y. Aikawa 1995 Homogeneity of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. *J. Biochem.* **117**: 467–470

Hiromasa, Y., Y. Aso, S. Yamashita, Y. Aikawa and M. Ishiguro 1997 Further studies on thermal denaturation of pyruvate dehydrogenase complex from *Bacillus stearothermophilus*. *Biosci. Biotechnol. Biochem.* **61**: 1126–1132

Hiromasa, Y., Y. Aso, K. Mayanagi, Y. Inoue, T. Fujisawa, K. Meno and T. Ueki 1998a Guanidine hydrochloride– induced changes of the E2 inner core of the *Bacillus stearothermophilus* pyruvate dehydrogenase complex. *J. Biochem.* **123**: 564–567

Hiromasa, Y., Y. Aso, S. Yamashita and T. Ueki 1998b Guanidine–hydrochloride induced denaturation of dihydrolipoamide dehydrogenase. *Protein Sci.*, Suppl. 1, **7**: 106

Hiromasa, Y., Y. Aso, S. Yamashita and K. Meno 2000 Thermally induced disintegration of the *Bacillus stearothermophilus* dihydrolipoamide dehydrogenase. *Biosci. Biotechnol. Biochem.* **64**: 1923–1929

Izard, T., A. Ævarsson, M. D. Allen, A. H. Westphal, R. N. Perham, A. D. Kok, and W. G. Hol 1999 Principles of quasi–equivalence and Euclidean geometry govern the assembly of cubic and dodecahedral cores of pyruvate dehydrogenase complexes. *Proc. Natl. Acad. Sci. USA*, **96**: 1240–1245

Jaenicke, R. and R. N. Perham 1982 Reconstitution of the pyruvate dehydrogenase multienzyme complex from *Bacills stearothermophilus*. *Biochemistry*, **21**: 3378–3385

Lessard, I. A. D., G. J. Domingo, A. Borges and R. N. Perham 1998 Expression of genes encoding the E2 and E3 components of the *Bacillus stearothermophilus* pyruvate dehydrogenase complex and the stoichiometry of subunit interaction in assembly *in vitro*. *Eur. J. Biochem.*, **258**: 491–501

McCartney, R. G., S. J. Sanderson and J. G. Lindslay 1997 Refolding and reconstitution studies on the transacetylase–protein X (E2/X) subcomplex of the mammalian pyruvate dehydrogenase complex: evidence for specific binding of the dihydrolipoamide dehydrogenase component to sites on reassembled E2. *Biochemistry*, **36**: 6819–6826

Pace, C. N., B. A. Shirley and J. A. Thomson 1989 Measuring the conformational stability of a protein. In "Protein structure", ed. Creighton, T. E. IRL Press, Oxford, pp. 311–330

Packman, L. C., R. N. Perham and G. C. K. Roberts 1984 Domain structure and ^{1}H–n.m.r. spectroscopy of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*. *Biochem. J.*, **217**: 219–227

Perham, R. N. 1996 Interaction of protein domains in the assembly and mechanism of 2–oxo acid dehydrogenase multienzyme complexes. In "Alpha–keto acid dehydrogenase complexes", eds. Patel, M. S., Roche, T. E., and Harris, R. A., Birkhaeuser Verlag, Basel, pp. 1–15

Perham, R. N. 2000 Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. *Annu. Rev. Biochem.*, **69**: 961–1004

Roche, T. E., S. Liu, S. Ravindran, J. C. Baker and L. Wang 1996 Role of the E2 core in the dominant mechanisms of regulatory control of mammalian pyruvate dehydrogenase complex. In "Alpha–keto acid dehydrogenase complexes", eds. Patel, M. S., Roche, T. E., and Harris, R. A., Birkhaeuser Verlag, Basel, pp. 33–52

West, S. M., J. E. Rice, E. S. Beaumont, S. M. Kelly, N. C. Price and J. G. Lindsay 1995 Dissociation and unfolding of the pyruvate dehydrogenase complex by guanidinium chloride. *Biochem J.*, **308**: 1025–1029

Witzmann, S. H. and Bisswanger 1998 The pyruvate dehydrogenase complex from thermophilic organisms: thermal stability and re–association from the enzyme components. *Biochim. Biophys. Acta*, **1385**: 341–352

Zhou, Z. H., W. Liao, R. H. Cheng, J. E. Lawson, D. B. McCarthy, L. J. Reed and J. K. Stoops 2001 Direct evidence for the size and conformational variability of the pyruvate dehydrogenase complex revealed by three–dimensional electron microscopy. The "breathing" core and its functional relationship to protein dynamics. *J. Biol. Chem.*, **276**: 21704–21713