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p-Chloromercuribenzoate-Induced Inactivation and Partial Unfolding of Porcine Heart Lactate Dehydrogenase

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Abstract—Purified porcine heart lactate dehydrogenase was inactivated and partially unfolded with *p*-chloromercuribenzoate (pCMB). With the increase of pCMB/enzyme ratio the enzyme was gradually inhibited till almost completely inactivated at the pCMB/enzyme ratio of 20 : 1. Native polyacrylamide gel electrophoresis showed that with the increase of pCMB/enzyme ratio the bands of native enzyme decreased till completely vanished. Meanwhile inactive multiple bands emerged and became thicker, which implied that lactate dehydrogenase became loose. The conformational changes of the enzyme molecule modified with pCMB were followed using fluorescence emission, ultraviolet difference, and circular dichroism (CD) spectra. Increasing pCMB concentration resulted in the decrease of fluorescence emission intensity. The ultraviolet difference spectra of the enzyme modified with pCMB exhibited an increasing absorbance in the vicinity of 240 nm with the increasing concentration of the inhibitor. The changes of the fluorescence and ultraviolet difference spectra reflected the conformational changes of the enzyme. The CD spectrum changes of the enzyme showed that its secondary structure changed as well. These results suggest that pCMB not only inhibits this enzyme but also influences its conformation (partial unfolding).

Key words: lactate dehydrogenase, *p*-chloromercuribenzoate, inactivation, unfolding

Lactate dehydrogenase (LDH, EC 1.1.1.27) is a tetrameric regulatory enzyme that catalyzes the reversible dehydrogenation of lactate, converting it to pyruvate. In this reaction, NAD^+ is used as the hydrogen acceptor. Although at least three different types of LDH subunits (M, H, and X) are known in vertebrates, the X subunits are usually present in only one, or at most a few tissues [1, 2]. The stereospecific NAD-dependent D-lactate dehydrogenase, which shares no homology with L-lactate dehydrogenase, catalyzes the reversible reduction of pyruvate into D-lactate [3, 4].

It is well known that each pig heart LDH subunit contains five cysteine residues [5], and one (Cys165) is essential for its activity. Modification of these groups by N-ethylmaleimide [6, 7] or *p*-chloromercuribenzoate (pCMB) [8, 9] leads to inactivation. Previous reports show that modification of the essential thiol group with small reagents alters neither the binding of NADH nor its fluorescence [6, 7].

pCMB is a specific and sensitive reagent for reaction with thiol group resulting in inactivation of many enzymes [10, 11]. pCMB treatment may result in conformational changes of the modified enzyme, which may lead to its activation and inactivation, as investigated with cytoplasmic aldehyde dehydrogenase from sheep liver [12].

In this paper, we examined the effect of pCMB on LDH. The results indicated that pCMB influenced not only the activity but also the structure of LDH, and that the enzyme underwent conformational changes and partial unfolding after modification.

MATERIALS AND METHODS

The porcine lactate dehydrogenase was prepared as described by Pesce et al. [13]. The final preparations were homogeneous on polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. NADH, pyruvate, NAD^+, pCMB, and 1-anilino-8-naphthalene sulfonate (ANS) were Sigma (USA) products. Centriprep YM-3 centrifugal filter unit was a Millipore (USA) product. Acrylamide and tetramethylethylenediamine were from Daiichi Pure Chemicals (Japan). Methylene-bis-acryl-

Abbreviations: LDH) lactate dehydrogenase; pCMB) *p*-chloromercuribenzoate; SDS) sodium dodecyl sulfate; PAGE) polyacrylamide gel electrophoresis; CD) circular dichroism; ANS) 1-anilino-8-naphthalene sulfonate.

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amide was from BDH Chemicals (New Zealand). Ammonium persulfate was from Wako Pure Chemicals (Japan). SDS was from Bio-Rad (USA). All other reagents were local products of analytical grade, used without further purification.

Enzyme concentration was determined by measuring the absorbance at 280 nm using the absorption coefficient $A^{1\%}_{1\,cm} = 14.0$ [14]. Enzyme activity was determined at 30°C by measuring the absorbance change at 340 nm accompanying the oxidation of NADH and using the molar absorption coefficient $\varepsilon_{340} = 6.23 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ as reported by Kornberg [15]. The reaction system contained 0.25 mM of NADH, 0.7 mM of sodium pyruvate, and 0.1 M phosphate buffer, pH 7.5. Staining of electrophoretic gels for activity was carried out as described previously [16]. Enzyme concentration and activity were determined with a Specord 200 UV VIS analytical spectrophotometer (Jena, Germany). All pH determinations were carried out at room temperature (25°C) on an Orion Model 720A pH-meter (USA).

Modification of LDH was carried out for 1 h at room temperature (25°C) with different pCMB/enzyme ratios from 4 : 1 to 100 : 1. Enzyme concentration was 6.3 μM in 0.01 M phosphate buffer (pH 6.5). Modified enzyme solutions were used for assay, electrophoresis, and for measurement of the fluorescence, circular dichroism (CD), and ultraviolet difference spectra. Electrophoresis of modified enzyme was carried out by PAGE in 0.025 M Tris/glycine buffer (pH 8.3) with 3.75% polyacrylamide for the stacking gel (pH 6.8) and 7.5% polyacrylamide for the separation gel (pH 8.9). SDS-PAGE was carried out according to the method of Laemmli [17]. Ultraviolet differential spectra were measured on the same Specord 200 spectrophotometer with two cell holders. The cuvette in the reference beam compartment contained native enzyme solution. Unbound pCMB solution was separated from modified enzyme solution using Centriprep YM-3 membrane (mol wt. cutoff 3000) by centrifuging at 3000*g* for 30 min at 4°C, before each difference absorption spectrum was measured. Fluorescence spectra were measured with a Hitachi 850 spectrofluorometer (Japan). The excitation wavelength was 280 nm for intrinsic fluorescence and 380 nm for ANS-binding fluorescence. A Jasco 500C CD spectropolarimeter (Jasco Research Ltd., Canada) was used for CD measurements in the far-ultraviolet region from 200 to 250 nm. All the measured spectra were corrected spectra.

All measurements were carried out in 0.01 M phosphate buffer (pH 6.5) at 25°C.

RESULTS

Inactivation of porcine lactate dehydrogenase by pCMB. The effect of pCMB on the activity of LDH has been studied. Figure 1 shows the residual activity of modified enzyme with different doses of pCMB. When the mole ratio of pCMB/enzyme was up to 20 : 1, the enzyme lost its activity almost completely. The inactivation was a two-stage process. The first rapid stage finished in a few minutes and the second slow stage finished in 1 h or a bit longer (data not shown).

Electrophoresis of modified enzyme. The modified enzyme was subjected to native PAGE and SDS-PAGE. Figure 2 shows the electrophoresis patterns of the enzyme modified with different concentrations of pCMB. Multi bands emerged in every lane except the one for the enzyme without pCMB. With the increase of inhibitor concentration, the bands of native enzyme became thin-

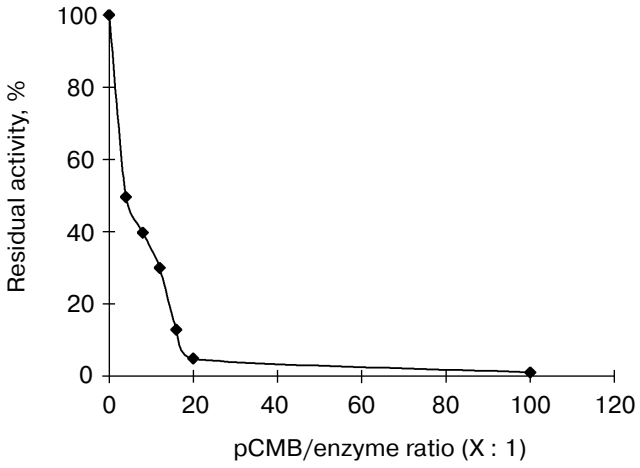

Fig. 1. Inactivation curve of LDH modified with different pCMB/enzyme ratios. The enzyme was incubated for 1 h at room temperature (25°C) in 0.01 M phosphate buffer (pH 6.5) with different ratios of pCMB.

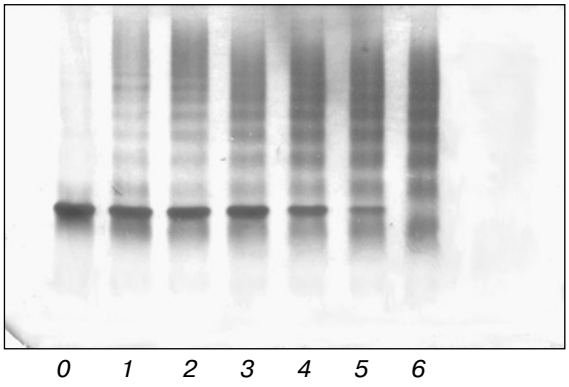

Fig. 2. Native PAGE of modified LDH: *0*) enzyme without pCMB; *1-6*) enzyme modified with pCMB at various pCMB/enzyme ratios: 4 : 1, 8 : 1, 12 : 1, 16 : 1, 20 : 1, and 100 : 1, respectively.

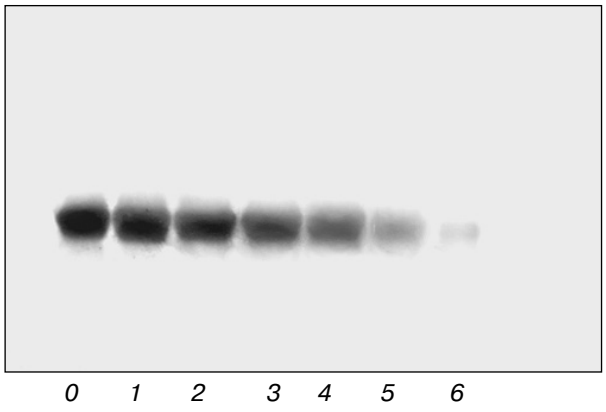

Fig. 3. Staining for activity of modified LDH. The labeling is as for Fig. 2.

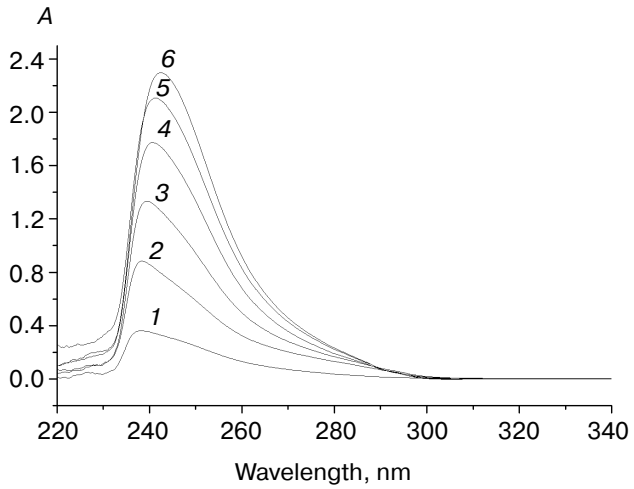

Fig. 4. Differential absorption spectra of modified LDH. The pCMB/enzyme ratios for curves *1-6* were 4 : 1, 8 : 1, 12 : 1, 16 : 1, 20 : 1, and 100 : 1, respectively.

ner till almost disappeared at the pCMB/enzyme ratio of 20 : 1. In the meantime, the multiple bands became thicker, which implied the native enzyme became loose due to the reaction with the inhibitor. The concentration of each band increased almost in parallel with pCMB concentration, which indicated that the modified enzyme could be relatively stable in different conformations and these different conformations formed and reached to a relatively stable balance rapidly after the enzyme was modified. Activity staining in Fig. 3 showed that the multiple bands had no activity. SDS-PAGE showed that there was only one band in the respective lane for LDH modified with different concentrations of pCMB, their positions were the same as that of the native enzyme (data not shown). This result indicated that the multiple bands were made up of full-length LDH subunits.

Differential absorption spectra of modified enzyme. The effect of pCMB concentration on the ultraviolet differential spectra of LDH is shown in Fig. 4. The enzyme modified with pCMB produced a positive peak in the vicinity of 240 nm. When pCMB concentration increased further, the peak increased in magnitude, and the maximum absorbance had a slight red shift from 238 to 242 nm.

Conformational changes of the enzyme modified with pCMB. Changes in the intrinsic protein fluorescence emission spectra of LDH, which reacted with pCMB of different ratios, are shown in Fig. 5. Increase in the pCMB ratio caused a marked decrease in the fluorescence emission intensity, but no obvious fluorescence shift was observed. A control experiment showed that free pCMB had little effect on the fluorescence emission intensity of the enzyme within the range of pCMB concentrations employed in the present investigation.

The fluorescence emission of ANS is known to increase when the dye binds to the hydrophobic regions of a protein [18]. Figure 6 shows the fluorescence emission spectra of ANS in the absence and presence of pCMB. ANS fluorescence emission spectrum of LDH shows a peak around 461 nm. pCMB modification had little influence on the peak position, but greatly influenced its intensity. Increasing pCMB ratio resulted in marked increase of LDH-bound ANS fluorescence emission intensity. Control experiments showed that pCMB had little effect on the fluorescence property of ANS within the pCMB concentration range used in the present work.

Figure 7 shows the CD spectra of LDH modified with different pCMB/enzyme ratios. The data showed a clear loss in secondary structure in modified LDH, which indicated that the conformation of the enzyme became loose after the treatment. And with the increase of pCMB/enzyme ratio, the loss in secondary structure increased.

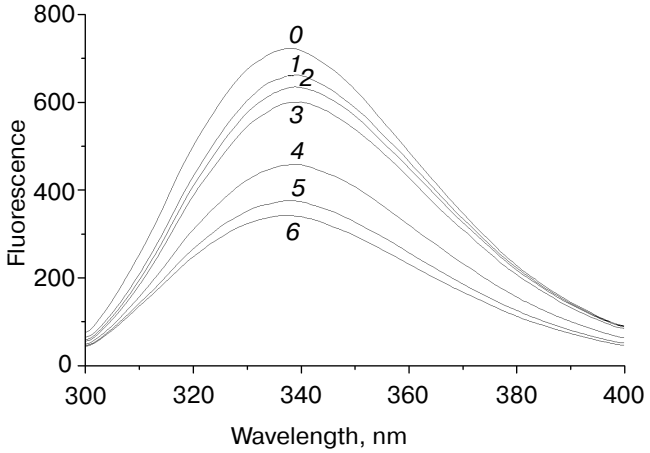

Fig. 5. Intrinsic fluorescence emission spectra of modified LDH. The pCMB/enzyme ratios for curves *1-6* were 4 : 1, 8 : 1, 12 : 1, 16 : 1, 20 : 1, and 100 : 1, respectively. Curve *0* was for the enzyme solution without pCMB.

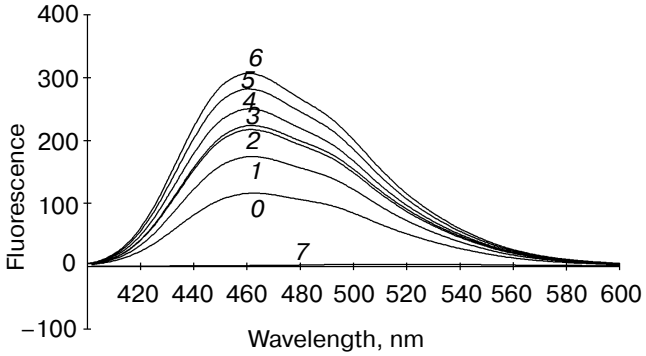

Fig. 6. ANS-binding characteristics of modified LDH. The final concentration of ANS was 94.5 μM. The pCMB/enzyme ratios for curves *1-6* were 4 : 1, 8 : 1, 12 : 1, 16 : 1, 20 : 1, and 100 : 1, respectively. Curve *0* was for the enzyme without pCMB and curve *7* for the solution with the same ANS concentration without enzyme. The excitation wavelength was 380 nm.

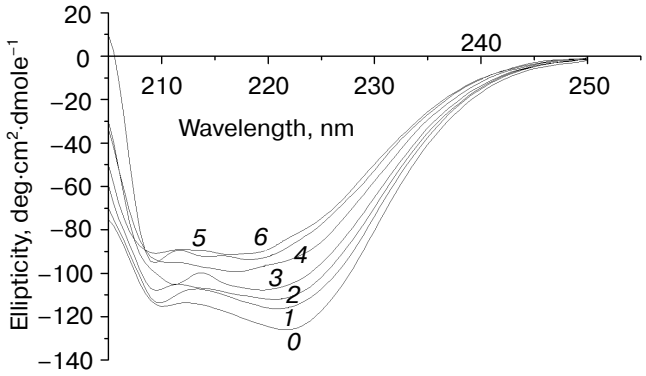

Fig. 7. CD spectra of modified LDH. The pCMB/enzyme ratios for curves *1-6* were 4 : 1, 8 : 1, 12 : 1, 16 : 1, 20 : 1, and 100 : 1, respectively. Curve *0* was for the enzyme without pCMB.

DISCUSSION

Organic mercurial compounds are specific and sensitive reagents available for reaction with sulfhydryl groups, and they have been widely used in studies of the biochemical function of sulfhydryl groups. In the present investigation, treatment with pCMB not only resulted in inactivation of LDH but also resulted in conformational changes and its partial unfolding. The conformational changes of LDH modified with pCMB were accompanied with inactivation. There was a balance between different conformations of modified enzyme. With the use of SDS-PAGE, it was verified that the multiple bands with lower mobilities than that of native LDH on native PAGE after modification were made up of full-length LDH subunits. No molecular weight band lower than that of LDH subunit was found on SDS-PAGE, which implied that no rupture happened in the polypeptide chain after modification. Thus it was supposed that the multiple bands be mainly made of modified LDH in different conformations.

The ultraviolet differential spectra drew out the difference between modified and native enzyme. The increase of absorbance around 240 nm was due to mercaptide formation [19], and the slight shift of the peak also reflected the conformation changes of the modified enzyme. Intrinsic fluorescence showed that the enzyme became loose after being treated with pCMB. ANS-binding fluorescence indicated that more hydrophobic groups were exposed with the increase of pCMB/enzyme ratio. CD results also provided the information that modified enzyme became loose and partially unfolded and the extent of the change was in proportion to the ratio of pCMB/enzyme. On the whole, all spectral data gave information that the modified enzyme underwent conformation changes and partial unfolding after modification, which was in accord with the results of PAGE.

There are five cysteine residues in each pig heart LDH, these being Cys36, Cys133, Cys165, Cys187, and Cys291 [5]. They are distributed in different structures—α-helix, β-sheet, or the connecting loops. Cys165 is known to be relevant to activity and is located at the distance of 10 Å from the substrate-binding site [3]. Other Cys residues, such as Cys291, are involved in the interaction between subunits. The present investigation shows that the enzyme was partially unfolded after these Cys residues reacted with pCMB, which implied that their modification might influence the structural stability of the enzyme.

Other researchers have also reported the influence of pCMB on conformation of oligomeric proteins. Rosemary et al. [12] showed that pCMB could also influence the conformation of modified enzyme, which might lead to its activation and inactivation, as investigated with cytoplasmic aldehyde dehydrogenase from sheep liver. But no in-depth study of conformational changes of the pCMB-treated enzyme has been reported. The present investigation stresses this effect of pCMB, as discussed above: pCMB not only inactivated LDH but also partially changed the conformation and structure of the enzyme.

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