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STUDIES ON CHICKEN LIVER XANTHINE DEHYDROGENASE WITH REFERENCE TO THE PROBLEM OF NON-EQUIVALENCE OF FAD MOIETIES

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Summary

1. Reduction of chicken liver xanthine dehydrogenase (xanthine : NAD^+ oxidoreductase, EC 1.2.1.37) by xanthine under anaerobic condition proceeded in two phases. This biphasicity may be due to functional and non-functional enzymes in the enzyme preparation.

2. Cyanolysis of a persulfide group of chicken liver enzyme resulted in an inactivation of the enzyme. The non-functional enzyme in the standard enzyme preparation was found to lack persulfide groups at the active sites.

3. The remaining NADH-Methylene Blue oxidoreductase activity, after KI treatment of the xanthine-reduced enzyme of a high flavin activity ratio, is not at the level of 50% of the initial activity, differing from the report suggesting non-equivalence of FAD chromophores.

4. The findings in the present report indicate that FAD chromophores of chicken liver enzyme are essentially equivalent.

Introduction

The presence of non-functional enzyme in milk xanthine oxidase, containing a full complement of molybdenum, flavin and iron-sulphur prosthetic groups has been revealed in the last few years [1,2], The eralier finding [3] of the non-equivalence of flavin chromophores of milk xanthine oxidase has been shown to be due to the presence of this non-functional enzyme in the enzyme preparation. Subsequent investigations by Massey et al. [4,5] have revealed that the lability of a persulfide group required for catalysis is responsible for the non-functional enzyme. Very recently, a persulfide group has been reported to be present also in the related metalloflavoproteins, i.e. rabbit liver aldehyde oxidase [8] and turkey liver xanthine dehydrogenase [6].

On the other hand, Kanda et al. [9] have reported that the preparation of chicken liver xanthine dehydrogenase, which contains a full complement of prosthetic groups, exhibits properties which indicate non-equivalence of the enzymic FAD moieties. They also suggested the possibility of the non-equivalence of the two FAD chromophores of this enzyme molecule. However, the possibility that the enzyme preparation used by Kanda et al. contains non-functional enzyme has been suggested by Nishino, who has obtained a highly active enzyme preparation [10].

This report indicates that the chicken liver xanthine dehydrogenase is similar to milk xanthine oxidase and other related metalloflavoproteins in the presence of non-functional enzyme, which is absent of active site persulfide.*

Materials and Methods

Liver xanthine dehydrogenase was purified from chickens fed a high protein diet by the previous method [10]. The enzyme preparations with various activity flavin ratio value were obtained in the different batches of preparation. Enzyme preparations for some experiments were partially inactivated by cyanide treatment. Cyanide treatment of the enzyme was performed at room temperature in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 5 mM KCN, and the reaction was stopped at the desired level of inactivation by passing through a Sephadex G-25 column.

Xanthine was obtained from Nutritional Biochemical Co., NAD^+ from Oriental Co. and NADH from Sigma. Other reagents were analytical grade.

Radioactive potassium cyanide having a specific activity of 15.0 Ci per mol was obtained from Daiichi Pure Chemical Co. This was diluted to a specific activity of 3.16 Ci per mol when used. Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, after mixing the solution with a Bray's scintillation fluid.

Absorption spectra and difference absorption spectra were recorded on a Cary Model 17 spectrophotometer.

Enzyme assays were carried out at 25°C in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The value of flavin activity ratio was obtained by the previous method [10].

During the treatment with KI, the oxidations of xanthine (0.15 mM) by NAD^+ (0.5 mM) and 2,6-dichlorophenol-indophenol (0.05 mM) were determined by measuring absorption change at 340 nm and 600 nm respectively. The oxidation of NADH (0.05 mM) by Methylene Blue (0.02 mM) was also determined spectrophotometrically at 340 nm.

Results and Discussion

Reduction of chicken liver xanthine dehydrogenase by xanthine

In 1952, Morell showed that the reduction of milk xanthine oxidase by xanthine under anaerobic condition, proceeded in two phases, the initial rapid

* Part of this work has been published in the form of an abstract [11].

phase being followed by a much slower one. He suggested that such biphasicity was due to the presence of functional and non-functional enzymes [12]. This result was confirmed in the subsequent investigation [13,6]. Recently a similar result has been reported by Branzoli et al. with rabbit liver aldehyde oxidase [8].

The reduction of the standard enzyme preparation of chicken liver xanthine dehydrogenase by xanthine under anaerobic condition displayed also a biphasic course. When the enzyme preparation with an activity flavin ratio at 25°C of 283 was reduced anaerobically by 0.2 mM xanthine, the initial rapid decrease and a following slower decrease of absorbance at 450 nm were observed. After 2 or 3 h of the reaction, no further detectable absorption change was observed (Fig. 1a). A total 54% bleaching of the absorbance at 450 nm was observed, 85% of which was done at the initial phase.

In the enzyme preparations obtained by cyanide treatment, the activity flavin ratio was found to have a linear correlation with the extent of the initial rapid decrease in the absorbance at 450 nm (Fig. 1b). Based on this result, activity flavin ratio value of the fully functional enzyme is calculated to be approximately 350 at 25°C. This value is considerably higher than that of milk xanthine oxidase reported by McGartoll et al. [2] and Massey et al. [15]. This value may not, however, be so different from that of turkey xanthine dehydrogenase, calculated from the value obtained by measuring activity at 340 nm reported by Cleere et al. [7].

Cyanolysis of active site persulfide

Incubation of chicken liver xanthine dehydrogenase with cyanide resulted in characteristic alterations in the absorption spectrum of the enzyme. The

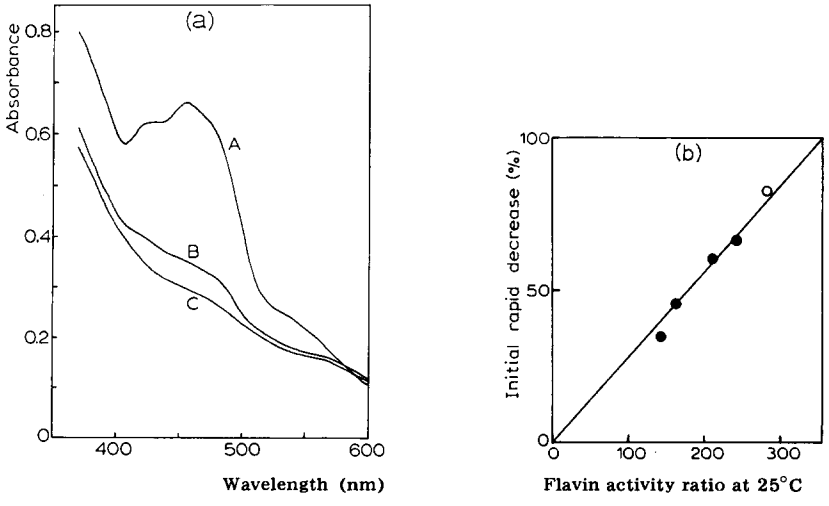

Fig. 1 (a) Absorption spectra of chicken liver xanthine dehydrogenase in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. A, oxidized enzyme; B, immediately after mixing the enzyme with xanthine under anaerobic conditions; and C, 3 h after mixing the enzyme with xanthine under anaerobic conditions. (b) Correlation of the extent of initial rapid decrease of absorbance at 450 nm by xanthine versus the flavin activity ratio. (○) prepared naturally by standard purification method; (●) prepared by cyanide treatment.

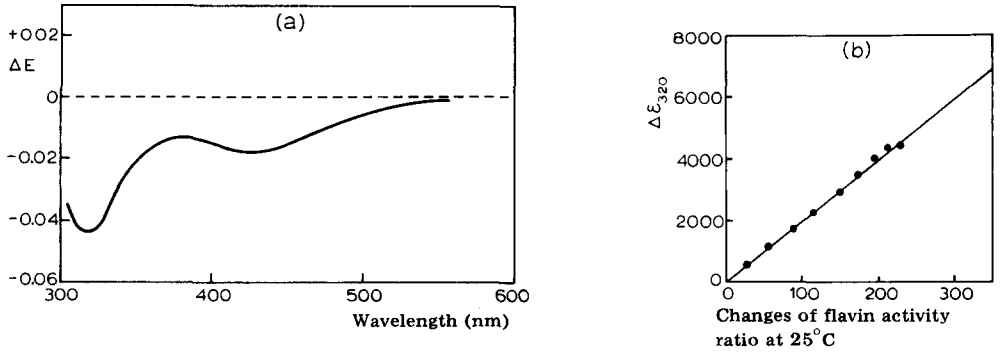

Fig. 2 (a) Difference spectrum between cyanide-inactivated and native chicken liver xanthine dehydrogenase (final E_{450}, 0.38). (b) Correlation between the extent of the extinction coefficient change at 320 nm during the reaction of chicken liver xanthine dehydrogenase with cyanide and the changes of flavin activity ratio.

Enzyme (final E_{450}, 0.38) was incubated at room temperature in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 5 mM KCN. Absorbance changes at 320 nm were followed with a Cary Model 17 spectrophotometer and aliquots were taken for assay at various intervals.

difference spectrum (Fig. 2a) between cyanide inactivation and native chicken liver xanthine dehydrogenase is very similar to that of milk xanthine oxidase reported previously by Coughlan et al. [14] and Massey et al. [4]. As previously reported by Coughlan et al., the cyanide inactivation of the enzyme can be correlated with the change in absorbance at 320 nm [14]. Fig. 2b shows the proportional relationship between the decrease of the extinction at 320 nm of cyanide treated enzyme at various incubation times and the decrease of the flavin activity ratio. The molar extinction change at 320 nm was found to be 4450 per equivalent of flavin for the enzyme preparation with a flavin activity ratio of 233. This is equivalent to an extrapolated value of 6900 for fully functional enzyme (activity flavin ratio of 350), close to that obtained with milk xanthine oxidase [4] and rabbit liver aldehyde oxidase [8].

Because of the similarities in the alteration of the absorption spectrum by cyanide treatment among milk xanthine oxidase, aldehyde oxidase and chicken liver xanthine dehydrogenase, it seems possible that the cyanide inactivation of the chicken liver xanthine dehydrogenase might be a consequence of the cyanolysis of the active site persulfide.

Accordingly, the amount of thiocyanate produced on inactivation by cyanide of chicken liver xanthine dehydrogenase was determined. The normally prepared enzyme preparation of a low flavin activity ratio (233, 67% functional) was incubated with radioactive cyanide. After complete inactivation, followed by the addition of 1 mg of non-radioactive ammonium thiocyanate as carrier the preparation was passed through a Sephadex G-25 column (1 × 60 cm). The inactivated enzyme was eluted at a volume of 30 ml and the unreacted cyanide at a volume of 68 ml. The elution of thiocyanate at a volume of 91 ml was detected with 5% $Fe(NO_3)_3$ in 25% HNO_3. The fraction of highest concentration of thiocyanate was rechromatographed on the same column. The radioactivity of the fraction having the highest concentration of thiocyanate was determined. The amount of radioactive thiocyanate released

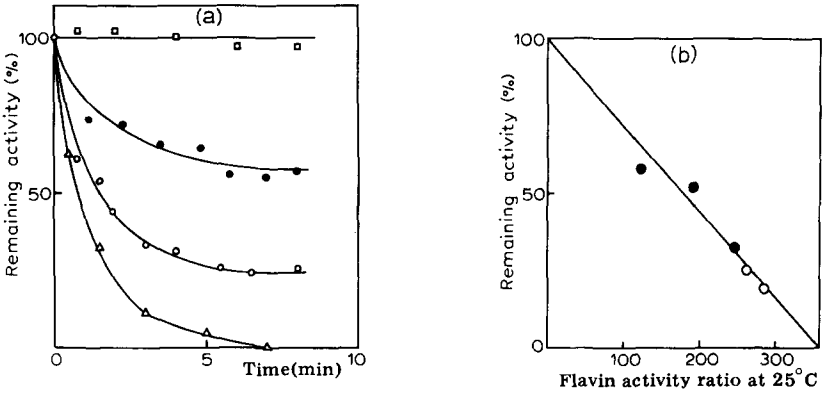

Fig. 3 (a) Effect of KI on the catalytic properties of chicken liver xanthine dehydrogenase. The enzyme was incubated with 0.2 mM xanthine in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA at room temperature. After 1 min, the reaction mixtures were cooled to 0°C and then exposed to 3 M KI. Aliquots were subjected to the measurements of the following activities : (□), xanthine-2,6-dichlorophenol-indophenol; (△), xanthine-NAD^+; (○), NADH-Methylene Blue (original flavin activity ratio; 263); (●), NADH-Methylene Blue (original flavin activity ratio; 121). The abscissa represents the time of incubation with KI. (b) Correlation between the extent of residual NADH-Methylene Blue activity after KI treatment and the flavin activity ratio of the various enzyme preparations. (○) prepared naturally with standard purification method; (●) prepared by cyanide treatment.

and calculated from the recovery of carrier thiocyanate, was 0.66 mol per mol of enzyme-bound FAD.

KI treatment of chicken liver xanthine dehydrogenase

Fig. 3a shows the effect of KI treatment on various activities of the purified chicken liver xanthine dehydrogenase prereduced with xanthine. Irrespective of the enzyme preparations used, oxidation of xanthine by 2,6-dichlorophenol-indophenol was essentially unaffected, whereas that by NAD^+ was rapidly abolished by KI treatment. When the xanthine-reduced enzyme of a flavin activity ratio of 263 was treated with KI, NADH-Methylene Blue oxidoreductase activity was rapidly lost and attained a steady lower level. The remaining activity was not at the level of 50% of initial activity as reported by Kanda et al. [9], but at a level of less than 30%.

For the enzyme preparations of various flavin activity ratios, a linear correlation was found between the flavin activity ratio and the remaining NADH-Methylene Blue activity after treatment of the xanthine-reduced enzyme with KI (Fig. 3b). Thus the loss of 50% of flavin moiety of the xanthine-reduced enzyme by KI treatment described by Kanda et al. [9], might be due to the presence of 50% non-functional enzyme in the preparation they used, rather than the non-equivalence of the flavin chromophore.

Previous work has suggested the possibility of the presence of a non-functional enzyme which contains a full complement of molybdenum, flavin and iron-sulphur prosthetic groups in the enzyme preparation used by Kanda et al.. The results presented above support this suggestion and further emphasize the similarities between the presence of a non-functional enzyme missing a persulfide group at the active site in milk xanthine oxidase and in other related metalloflavoproteins.

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