Interaction between Rat Lactic Dehydrogenase M₄ Isozyme and Vitamin B₂ Derivatives

Kosuke YAMADA, Tadao HASEGAWA, and Takao SUZUKI¹

*Department of Agricultural Chemistry,
Tokyo University of Agriculture,
Sakuragaoka, Setagaya-ku, Tokyo 156, Japan*

(Received August 5, 1981)

Summary In an attempt to clarify the mechanism of LDH protein reaction with FMN, amino acid residues in the protein moiety such as histidine, arginine, tyrosine and tryptophan were chemically modified so that their effects on LDH activity and FMN reaction could be investigated. Although it was recognized that histidine and arginine residues participate in the catalytic action, and that these residues exist at the binding site of coenzyme and substrate, no reaction of FMN with these residues was noted. LDH was slightly inactivated when tryptophan residue was modified, but inhibition of this activity was completely prevented when FMN was allowed to react with zymoprotein in advance. As is true with native LDH, LDH with a modified histidine residue or tyrosine residue reacted with FMN, but LDH with a modified tryptophan residue did not so react. It was therefore shown that the tryptophan residue of native LDHM₄ zymoprotein reacts with FMN.

Key Words lactic dehydrogenase M₄ isozyme, FMN, arginine, histidine, tryptophan, tyrosine, 2,3-butanedione, diethyl pyrocarbonate, tetranitromethane, diethyl(2-hydroxy-5-nitrobenzyl) sulfonium bromide

The authors previously reported (1) that LDHM₄ isozyme extracted from rat skeletal muscle reacts with either FMN or FAD. In the reaction of the reduction system generating lactate from pyruvate, enzymatic activity was activated when LDH was combined with FMN or FAD. In this paper, the results of our studies on the reaction of FMN with various amino acid residues contained in zymoproteins are discussed in order to clarify the interaction between LDHM₄ isozyme and FMN.

Amino acid residues contained in LDH zymoprotein were chemically modified in an attempt to investigate the activity of this modified enzyme and to compare it to FMN. Furthermore, the enzyme with modified histidine, tyrosine and tryptophan residues was allowed to react with FMN, and a reaction mixture was isolated by

¹ 山田貢佑, 長谷川忠男, 鈴木隆雄

starch gel electrophoresis to confirm whether or not FMN was responsible for the reaction and which one of the amino acids was related to FMN.

MATERIALS AND METHODS

1. *Starch gel electrophoresis.* LDH activity measurement and vitamin B₂ determination were carried out as already reported in previous papers (1-3).

2. *Purification of LDH M₄ isozyme.* Purification was performed using the method of Stolzenbach (4). The enzyme was purified according to the following order: crude extract, salting out by 70% ammonium sulfate, salting out by 25-50% ammonium sulfate and ion exchange chromatography with CM-Sephadex C-50. The final product was obtained as a white precipitate, and disc electrophoresis revealed a single band of the purified enzyme.

3. *Chemical modification of LDH zymoprotein.*

a) Modification of arginine residue: In the modification of arginine residue carried out by the method of Yang *et al.* (5), an enzyme dissolved in sodium borate buffer (pH 7.9) was added to 3.4 mM 2,3-butanedione solution at 25°C.

b) Modification of histidine residue: As with the method of Holbrook *et al.* (6), imidazol residue was allowed to react with 0.114 mM diethyl pyrocarbonate at room temperature by dissolving the enzyme in 0.1 M of pH 7.0 phosphate buffer solution.

c) Modification of tyrosine residue: Referring to the method of Riordan *et al.* (7), tetranitromethane at various concentrations was added to the enzyme dissolved in 0.05 M Tris buffer solution (pH 8.0) at room temperature.

d) Modification of tryptophan residue: Before modification using the method of Heilmann *et al.* (8) with dimethyl (2-hydroxy-5-nitrobenzyl)sulfonium bromide (Koshuland Reagent K-I WS, DHN-SB), DTNB was added to the enzyme dissolved in 50 mM triethanolamine containing 0.1 mM of EDTA pH 7.5 buffer solution in order to protect the SH groups contained in the zymoprotein. After reaction modified with DHN-SB was confirmed, thiol residues were regenerated by adding 20 mM 2-mercaptoethanol.

RESULTS

1) *Inactivation and retention of activity by chemical modification*

The LDH was markedly inactivated when arginine residues were modified with 2,3-butanedione. This inhibition was reduced by 50% when NADH was added prior to reaction, and also by 50% when the addition was made in the course of the reaction (after 20 min). Approximately 90% of the activity was retained by allowing sodium oxamate and NADH to coexist prior to the reaction (Fig. 1). The data on the trend of decline of the activity in Fig. 2 are plotted with the number of moles of the modified arginine residues as abscissa and the inhibition ratio as ordinate. This figure indicates that two arginine residues are essential to the activity of LDH

Fig. 1. Protection of LDHM₄ activity against inhibition by 2,3-butanedione in the ternary complex. The enzyme at a concentration of 0.5 mg/ml in 0.25 M borate buffer (pH 7.9) was treated with 2,3-butanedione at 25°C. ●, 3.4 mM 2,3-butanedione; ■, 0.33 mM NADH was added after 20 min; □, 0.33 mM NADH was added; ○, 0.33 mM NADH and 1 mM sodium oxamate were added.

Fig. 2. Remaining activity against moles of 2,3-butanedione bound per 1 mol of LDHM₄ subunit. Each experimental LDHM₄ was used at a concentration of 0.5 mg/ml. Reaction was carried out at 25°C in 0.25 M borate buffer (pH 7.9). Reaction times were 20 hr (●), 30 hr (▲), 48 hr (△), and 60 hr (○).

protein.

The LDH activity was completely lost by the modification of histidine residues with diethyl pyrocarbonate. This inhibition was retained to a level of about 40%

Fig. 3. Protection of LDHM₄ activity against inhibition with diethyl pyrocarbonate in the ternary complex. The enzyme in 0.1 M phosphate buffer (pH 6.0) was treated with diethyl pyrocarbonate at room temperature. ●, 0.114 mM diethyl pyrocarbonate; □, 1 mM NADH was added; ■, 1 mM sodium oxamate was added; ○, NADH and sodium oxamate were added.

Fig. 4. Remaining activity against moles of diethyl pyrocarbonate bound per 1 mol of LDHM₄ subunit. Reaction was carried out at 25°C in 0.1 M phosphate buffer (pH 6.0); reaction times were 0.5 min (●), 1 min (◇), and 2 min (○), respectively.

when either NADH or sodium oxamate alone was present in the reaction system in advance. The activity of enzyme incubated in the reaction system was almost completely retained when NADH and sodium oxamate coexisted (Fig. 3). In other words, these phenomena indicate that the histidine residues participate in the

Fig. 5. Effects on inactivation of LDHM₄ with DHN-SB, and influence of FMN. 0.5 mg/ml of the enzyme in 0.1 M phosphate buffer pH 7.5 (○) and in 0.01 M triethanolamine buffer pH 7.5 (●) was treated with DHN-SB at 25°C. (○) and (●), a solution of enzyme was treated with DHN-SB (0.014 mM); ◆, 0.5 mM FMN was mixed with the enzyme solution before treating with DHN-SB.

catalytic action. When the declining rate of the activity corresponding to the number of modified histidine residues in the protein moiety was studied in the same manner as shown in Fig. 2, it was recognized that a single histidine residue proved to be essential to the activity (Fig. 4).

Although it is not shown in the figure, the LDH was inhibited by about 60% when tyrosine residues in LDH were modified with tetranitromethane. However, the inhibition was weaker, and the activity was almost unaffected by tetranitromethane at different concentrations. No effects of protection against this inhibition were exhibited by NADH and sodium oxamate. Thus, no tyrosine residues participated in the enzyme activity. The LDH was inhibited when arginine, histidine and tyrosine were modified with 2,3-butanedione, diethyl pyrocarbonate and tetranitromethane, and the LDH activity was not at all affected by FMN and FAD previously added to the reaction system. This indicates that neither FMN nor FAD is related to the amino acid residues.

LDH was inhibited when tryptophan residues were modified with DHN-SB, but this inhibition was weak, at a level of approximately 20%. Similar results were obtained when two buffer solutions of phosphate and triethanolamine were used instead. Nonetheless, the activity of LDH bonded to FMN was not inhibited at all by the addition of DHN-SB. Therefore, the inhibitory effects on DHN-SB on the LDH activity were completely prevented because of the presence of FMN (Fig. 5).

2) Reaction of chemically modified LDHM₄ isozyme with FMN

In an attempt to determine which one of the amino acid residues of the LDH zymoprotein combines with FMN, LDH with various kinds of modified amino acid

Fig. 6. B₂ contents in the reaction products with FMN and LDHM₄ isozyme. A, native LDHM₄ isozyme; B, modified LDHM₄ isozyme with HNBB. Total of analytical values are shown as B₂ contents (μg) in 0.15 ml of reaction solution, containing 0.75 mg of native or modified proteins. Reaction was carried out at 37°C for 1 hr. Dialysis performed in a cold room (4°C) overnight.

residues was allowed to react with FMN, and the relationships between the amounts of the native LDH having reacted with FMN were compared. The amounts of the native LDH reacting with FMN are given in Fig. 6A. According to the results obtained, 13 μg of FMN reacted per mg of protein, and this value corresponds to what was described in the previous report (*1*), since 3.65 mol of FMN were bound to 1 mol of LDH zymoprotein.

Tyrosine residues of LDHM₄ were modified with tetranitromethane, and the modified protein was allowed to react with FMN. After reaction, the protein reacted with FMN was isolated by starch gel electrophoresis. When the amount of FMN bound to the modified protein was determined, 11.5 μg of FMN was found to have reacted with each 1 mg of protein. On the other hand, when LDH protein with histidine residues modified with diethyl pyrocarbonate was allowed to react with FMN, the amount of enzyme reacted was determined in the same manner, and was found to be 10.4 μg. Almost no difference was noted between these results and those of the reaction between native LDH not chemically modified and FMN.

Although LDHM₄ protein with tryptophan modified with DHN-SB was allowed to react with FMN, and the bound FMN weight was determined, FMN was found not to be electrophoretically situated at a position corresponding to that in a proteinic pattern (Fig. 6B). It may be explained from these results that FMN was not combined since tryptophan residues in LDHM₄ protein were blocked by the modification with DHN-SB.

DISCUSSION

Although the enzymatic activity of LDH was significantly inhibited or modification with 2,3-butanedione and diethyl pyrocarbonate, this inhibition was prevented when NADH and sodium oxamate were present in the reaction system. The facts are that arginine and histidine residues participate in the LDH activity, and that these residues are situated at the binding site of coenzyme and substrate.

However, since the presence of FMN does not affect the inhibition of the activity at all, both arginine and histidine residues are conceivably unrelated to FMN. On the other hand, it is also conceivable that tyrosine residues modified with tetranitromethane do not participate in the LDH activity. In our previous report (1), we referred to a hypothesis that aromatic amino acids may be related to the bonding between FMN and LDHM₄, but the existence of FMN did not in the least affect the inhibition caused by tetranitromethane. Thus, it is presumable that tyrosine residues in LDH protein do not participate in the binding with FMN.

The LDH was inhibited by about 20% when the modification was performed with DHN-SB, and this indicates that tryptophan residues are not directly related with LDH activity. Nevertheless, no inhibition was caused by DHN-SB in LDHM₄ protein-bound to FMN. Therefore, it may be concluded that FMN is significantly protective against the inhibition caused by DHN-SB. As was described in the previous report (1), the LDH activity was activated by about 45%, but it was also activated when FMN was added to the enzyme with the modified tryptophan residues. In our current experiments in which the enzyme with the modified amino acid residues was allowed to react with FMN, it was evidenced that both LDH with modified histidine residues and LDH with modified tyrosine residues were favorable to FMN. These results corresponded to those expected for the reaction of native LDHM₄ and FMN, although they indicate that histidine and tyrosine residues are not concerned with the bonding between LDHM₄ and FMN. However, as shown in Fig. 6B, the modified enzyme was not bound to FMN at all when LDH with tryptophan residues modified with DHN-SB was allowed to react with FMN. Thus, it may be interpreted that FMN was not combined since tryptophan residues in LDHM₄ protein were blockaded with DHN-SB. It was therefore suggested that FMN was bound to tryptophan residues in LDHM₄ protein. In view of the results obtained by visible ray absorption spectrum and fluorescence spectrum analysis of FMN, it was indicated that FMN had nothing to do with hydrophobic groups such as aromatic residues of LDH, and this corresponds to the results which we obtained.

REFERENCES

- 1) Yamada, K., Hasegawa, T., and Suzuki, T. (1979): Studies on reaction of rat lactic dehydrogenase with vitamin B₂ derivatives. *Nutr. Rep. Int.*, **20**, 171-178.
- 2) Suzuki, T., Yamada, K., and Hasegawa, T. (1972): Studies on interaction of thiamine or its related compounds with sarcoplasmic proteins (Preliminary Report). *Vitamins* (in Japanese), **46**, 179-184.
- 3) Yamada, K., Hasegawa, T., and Suzuki, T. (1977): Studies on reaction of sarcoplasm with iBuTDS. *Nutr. Rep. Int.*, **16**, 779-788.
- 4) Stolzenbach, F. (1966): Lactic dehydrogenase (Crystalline), in *Methods in Enzymology*, ed. by Colowick, S. P., and Kaplan, N. O., Vol. 9, Academic Press, New York, pp. 278-288.
- 5) Yang, P. C., and Schwert, G. L. (1972): Inactivation of lactic dehydrogenase by butanedione. *Biochemistry*, **11**, 2218-2224.

- 6) Holbrook, J. J., and Ingram, V. A. (1973): Ionic properties of an essential histidine residue in pig heart lactate dehydrogenase. *Biochem. J.*, **131**, 729-738.
- 7) Riordan, J. F., and Vallee, B. L. (1972): Nitration with tetranitromethane, in *Methods in Enzymology*, ed. by Hirs, C. H. W., and Timasheff, S. N., Vol. 25, Academic Press, New York, pp. 515-521.
- 8) Heilmann, H. D., and Pfeleiderer, G. (1975): On the role of tryptophan residues in the mechanism of action of glyceraldehyde-3-phosphate dehydrogenase as tested by specific modification. *Biochim. Biophys. Acta*, **384**, 331-341.