Note

Amino Acid Residues in the Allosteric Site of L-Lactate Dehydrogenase from *Bifidobacterium longum*

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L-lactate dehydrogenases (LDHs; EC 1.1.1.27) are found in vertebrates and various bacterial species. Most bacterial LDHs are allosteric enzymes activated by fructose 1,6-bisphosphate (FBP),¹⁾ while the enzymes from vertebrates show no dependence on this effector.²⁾ We have studied the allostericity of LDH from *Thermus*.³⁻⁶⁾ To elucidate the mechanism of activation of bacterial LDHs further, we newly purified an LDH from *Bifidobacterium longum*, which differs from *Thermus* in phylogenetic aspects and physiological conditions. *Bifidobacterium* is mesophilic and anaerobic, forms the predominant flora in human intestines, and is classified as an actinomycete. A FBP-dependent LDH from *B. bifidum* was reported,⁷⁾ but has not been purified.

Cells of *B. longum* aM101-2 were grown at 37°C in GAM broth (Nissui Pharmaceutical Co., Ltd.) for 35 h under an air atmosphere without agitation. A crude extract was obtained from 600 g (wet weight) of cells by the method described by Taguchi *et al.*³⁾ Tris-HCl buffer (20 mM, pH 7.0) was used throughout the operations described below. From the supernatant of 40% saturation of ammonium sulfate, an active fraction was separated by a column of Butyl-Toyopearl 650 M (Tosoh Corp.) with decreasing linear gradient of 40 to 0% saturation of ammonium sulfate. After dialysis, the enzyme was further purified by a column of 5'AMP-Sepharose 4B (Pharmacia LKB Biotechnology)⁸⁾ with an elution buffer containing 5 mM NAD⁺ and 1 M KCl, and that of Mono Q HR 5/5 (Pharmacia) with a linear gradient of 0 to 0.5 M KCl. LDH activity was assayed at 30°C by the method described by Taguchi *et al.*³⁾ The standard assay system for *B. longum* LDH contained 50 mM sodium Mes buffer (pH 6.0), 2 mM pyruvate, and 0.1 mM FBP. Protein was assayed by the procedure of Bradford.⁹⁾ The molecular weights of the enzyme and its subunit were measured by gel filtration

with a column of Superose 12 (Pharmacia)¹⁰⁾ and SDS-polyacryl amide gel electrophoresis (SDS-PAGE)¹¹⁾ using proteins in Combithek (Boehringer Mannheim GmbH) as molecular weight markers.

Two milligrams of the enzyme protein was digested by lysylendopeptidase (Wako Pure Chemical Co., Ltd.) in 1 ml of 50 mM Tris-HCl buffer (pH 8) for 24 hr. Digested peptides were treated with 0.1% EDTA and 70 mM 2-mercaptoethanol for 4 hr and with 70 mM moniodoacetate for 30 min. The sample was separated by reverse phase chromatography using an ODS-column, VP318-2251 (Senshu Science Co.), with an linear gradient of 0 to 60% acetonitrile containing 0.1% trifluoroacetic acid. Separated peptides were sequenced with a gas phase protein sequencer (Model 470A, Applied Biosystems).

The specific activity of the purified *B. longum* LDH was 828 U/mg. The molecular weight of the enzyme was 162,000 and 39,000 by gel filtration and SDS-PAGE, respectively (data not shown). The kinetic properties of *B. longum* LDH are listed in Table I. The enzyme had extremely low activity in the absence of FBP. Upon the addition of FBP, the enzyme activity was greatly increased. These results showed that the enzyme was a tetrameric allosteric enzyme like other bacterial LDHs. But, the concentration of FBP required for half-maximum activation for the enzyme was quite low compared to the values obtained in the case of other bacterial LDHs (0.6~4.6 μ M).^{3,12)}

Peptides obtained by proteolysis were separated into about 16 peaks by reverse phase chromatography. The N-terminal sequence of peak-16 was GATNYAIGM-SGVDIIEAVLHDTNRILPVSSM. This sequence corre-

Table I. SUMMARY OF THE KINETIC PROPERTIES OF *B. longum* aM101-2 LDH

Conditions for each assay are described in the table. The buffer was 50 mM sodium Mes (pH 6). (S)_{0.5} and (A)_{0.5} are the concentration for half-maximum activity of the substrate and FBP, respectively.

Conditions		
Optimum pH	6.0	0.2 mM NADH, 2 mM pyruvate, 0.5 mM FBP
K _m for NADH	8.8 μ M	pH 6, 8 mM pyruvate, 0.1 mM FBP
(S) _{0.5} for pyruvate	1.1 mM	pH 6, 0.2 mM NADH, 0.1 mM FBP
(A) _{0.5} for FBP	> 100 mM 26 nM	pH 6, 0.2 mM NADH pH 6, 0.2 mM NADH, 8 mM pyruvate

Abbreviations: LDH, L-lactate dehydrogenase; FBP, fructose 1,6-bisphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Table II. AMINO ACID RESIDUES OF *B. longum* AND OTHER LDHS CORRESPONDING TO THE RESIDUES FORMING A PART OF THE ANION-BINDING SITE IN VERTEBRATE ENZYMES^{5,14)}

LDHs from dogfish muscle (M4); pig muscle (M4); and heart (H4); *B. longum* (BL); *T. caldophilus* (TC); *Lactobacillus casei*; *Bacillus stearothermophilus* (BS). Numbering according to Eventoff *et al.*¹³⁾

Position	Residue						
	Dogfish M4	Pig		BL	TC	LC	BS
		M4	H4				
256	Asp	Asp	Asp	Asp	Arg	Arg	Arg
259	Glu	Glu	Glu	Glu	Arg	Lys	Arg
267	Arg	Arg	Arg	Arg	Gly	Ala	Ala
268	Val	Val	Ile	Ile	Val	Val	Ile
269	His	His	His	Leu	Tyr	Leu	Leu

sponds to sequences from residue 244 to 274 of other LDHs¹³⁾ and comprises a part of the amino acid residues found in the anion-binding site of vertebrate LDHs, as shown in Table II. The site has been thought to be the FBP-binding site of bacterial LDHs.¹⁴⁾ The charges of residues 256, 259, and 267 were conserved in all bacterial LDHs analyzed so far, but were different from those of vertebrate LDHs, and have been thought to be important in allosteric regulation of bacterial LDHs.^{5,12)} The corresponding amino acid residues of allosteric *B. longum* LDH were same as those of nonallosteric vertebrate enzymes. Charge properties in the FBP-binding site may not be the cause (s) of FBP-induced allosteric activation of bacterial LDHs and the mechanism of the phenomenon must be reinvestigated.

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