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L-Amino Acid Dehydrogenases in *Bacillus subtilis* Spores

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The presence of two kinds of L-amino acid dehydrogenase in resting spores of *Bacillus subtilis* was indicated. One of them was L-alanine dehydrogenase, which used only L-alanine as a substrate, and the other was nonspecific dehydrogenase, which used L-valine, L-isoleucine, L-leucine, and L-alanine (slightly) as substrates. Several properties of these dehydrogenases were compared.

It is well known that several amino acids have triggering action on spore germination (14), and that L-alanine especially acts as a trigger for spores of many kinds of *Bacillus* species (3, 6, 7, 12). Moreover, NAD (nicotinamide adenine dinucleotide)-linked L-alanine dehydrogenase has been purified from *Bacillus cereus* spores (10, 11), and a relationship between initiation of L-alanine-induced spore germination and L-alanine dehydrogenase was indicated.

In our laboratory it was shown that spores of *B. subtilis* (PCI 219) are able to germinate by L-valine or L-isoleucine as well as by L-alanine. If L-alanine dehydrogenase played an important role in L-alanine-induced spore germination, spores of this strain may have another dehydrogenase using valine or isoleucine as substrates in addition to L-alanine dehydrogenase.

In this investigation we describe the isolation and some characteristics of L-isoleucine dehydrogenase which has nonspecific action on L-valine, L-leucine, or L-alanine and a specific L-alanine dehydrogenase.

MATERIALS AND METHODS

Spore preparation. Spores of *B. subtilis* PCI 219, prepared on nutrient agar at 37 C for 5 days, were harvested and washed five times with sterilized distilled water by centrifugation at 1,400 × *g* for 30 min and kept frozen at −20 C until they were used. This preparation contained neither vegetative cells nor debris.

Measurement of spore germination. Spores preheated at 60 C for 30 min were suspended in sterilized 100 mM phosphate buffer (pH 7.2) containing individual L-amino acids in a final concentration of 50 mM (except that L-cystine, L-tryptophan, and L-tyrosine were in a final concentration of 5 mM), and were incubated at 37 C for 2 h. Germination was measured both as percentage of reduction in optical density (OD) at 650 nm determined with a Bausch & Lomb Spectronic 20 colorimeter (5) and as percentage of spores staining with 0.1% methylene blue. Initial OD values of the spore germination mixtures were about 0.35.

Crude enzyme preparation. Spores (10 g, wet weight) suspended in 50 mM phosphate buffer (pH 6.8) were disrupted with 30 g of 0.25- to 0.30-mm diameter glass beads by using a B. Braun cell homogenizer Model MSK for 1 h under cooling with dry ice, and the mixture was centrifuged at 10,500 × *g* for 30 min. The supernatant solutions were pooled and clarified by centrifugation at 100,000 × *g* for 1 h to remove particulate reduced nicotinamide adenine dinucleotide (NADH) oxidase (15). The supernatant fluid was used as the crude enzyme preparation.

Partial purification and isolation of each enzyme. Crude enzyme preparation was fractionated by the addition of varying amounts of solid ammonium sulfate. The ammonium sulfate precipitates were collected by centrifugation and dissolved in a minimal volume of 50 mM phosphate buffer (pH 6.8). These solutions were respectively added to the top of a Sephadex G-200 column (1.5 by 20 cm; bed volume 35 ml) previously equilibrated with 50 mM phosphate buffer (pH 6.8).

Isoelectric fractionation by electrolysis. Isoelectric fractionation was done by the method of Vesterberg et al. (16) for the isolation of L-isoleucine dehydrogenase and L-valine dehydrogenase from L-alanine dehydrogenase. An electrolysis column of 110-ml capacity with cooling mantle and platinum electrodes (LKB-Produkter) was used with electric power supply (Matsuyoshi Ikakikai Co.). The carrier ampholytes (LKB-Produkter) had isoelectric points distributed between pH 3 and 10, and were used at a final concentration of 2%. Crude enzyme preparation was introduced into the column as described by Vesterberg et al. (16), and electrolysis experiments were carried out at a temperature of 4 C for 24 h at a voltage of 900 V and a current of 3 mA. Fractions of 1.5 ml each were collected and the pH of each fraction was measured with a Hitachi-Horiba pH meter Model F-5.

Enzyme assays. Each L-amino acid dehydrogenase activity was measured spectrophotometrically at 340 nm at 37 C with each L-amino acid and nicotinamide adenine dinucleotide (NAD) as substrates, by using a cuvette of 1-cm light path. The reaction mixture

contained 300 μmol of carbonate-bicarbonate buffer (pH 10.1 or 10.7), 40 μmol of each L-amino acid, 1.2 μmol of NAD, enzyme plus additions as indicated, and distilled water to 3.0 ml. The specific activity was expressed as nanomoles of NAD reduced per minute per milligram of protein.

Measurement of protein. Protein was estimated by the method of Lowry et al. (9).

Molecular weight estimation by gel filtration. Assays were made by the method of Andrews (1). Partially purified enzyme solution (2.0 ml) containing 0.1 ml of 1% blue dextran 2,000, 0.2 ml of 30 mg of bovine serum albumin per ml, and 0.1 ml of 78,000 U of catalase per ml was applied to a column (1.5 by 80 cm; bed volume 141 ml) containing Sephadex G-200 suspended in 50 mM phosphate buffer (pH 6.8). Elution was accomplished by using the same buffer solution, and fractions of 3.5 ml each were collected. The elution volume was estimated from the absorption at the wavelength of 630 nm for blue dextran 2,000, 280 nm for bovine serum albumin, and from the decreased absorption of H_2O_2 at 240 nm for catalase activity (2).

Others. Optimal pH for the reaction catalyzed by each enzyme was examined by using tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer or carbonate-bicarbonate buffer. For heat inactivation studies various solutions of partially purified L-amino acid dehydrogenase were put into preheated carbonate-bicarbonate buffer and were placed in a water bath maintained at the desired temperature. The samples were removed and added directly to cuvettes containing the complete assay mixture minus enzyme.

The apparent Michaelis constants, K_m, were calculated from the plot according to Lineweaver and Burk (8). Effects of various metallic ions on each enzyme activity were investigated by adding 10 μmol of each metallic chloride to the complete assay mixture.

Chemicals. NAD was purchased from Boehringer Mannheim Co. All other chemicals including various L-amino acids were of the finest grade.

RESULTS

Germination of B. subtilis PCI 219 spores by various L-amino acids. Table 1 shows that 100% of the spores germinated in 100 mM phosphate buffer (pH 7.2) which was individually supplemented with L-alanine, L-valine, or L-isoleucine. Spore germination was also supported (30 to 50%) by L-leucine, L-cysteine, L-methionine, L-serine, or L-glutamic acid.

Each amino acid dehydrogenase activity in crude enzyme preparation obtained from spores. The crude enzyme preparation obtained from spores by sonic treatment and centrifugation showed high enzyme activity which deaminated L-alanine and moderate enzyme activities which deaminated L-isoleucine, L-valine, and L-leucine, but did not deaminate any other L-amino acid (Table 1).

TABLE 1. *Germination by various amino acids and presence of various L-amino acid dehydrogenase activities in crude enzyme preparation*

Amino acid	Spore germination (%)	Enzyme act.[a]
Glycine	0	0
L-Alanine	100	75.6
L-Valine	100	31.6
L-Leucine	30	37.0
L-Isoleucine	100	34.2
L-Phenylalanine	5	0
L-Tyrosine	0	0
L-Cysteine	50	0
L-Cystine	13	0
L-Methionine	30	0
L-Serine	30	0
L-Threonine	0	0
L-Proline	0	0
L-Hydroxyproline	10	0
L-Aspartic acid	0	0
L-Glutamic acid	35	0
L-Asparagine	4	0
L-Tryptophan	0	0
L-Histidine	0	0
L-Lysine	5	0

[a] Values of specific activity are expressed as nanomoles of reduced nicotinamide adenine dinucleotide per minute per milligram of protein.

On the basis of the data in Table 1, we considered two possibilities; one of them was that L-alanine dehydrogenase was able to use L-valine, L-isoleucine, and L-leucine as substrates besides L-alanine and the other was that there were several kinds of L-amino acid dehydrogenase in addition to L-alanine dehydrogenase.

Isolation of each amino acid dehydrogenase. The crude enzyme preparation was fractionated by the addition of varying amounts of solid ammonium sulfate to isolate L-valine dehydrogenase and L-isoleucine dehydrogenase from L-alanine dehydrogenase. As shown in Table 2, the most activities of L-valine dehydrogenase and L-isoleucine dehydrogenase appeared in 40 to 50% ammonium sulfate fractionation, whereas L-alanine dehydrogenase activity appeared in 50 to 70% ammonium sulfate fractionation. This fact suggests that there would be enzymes which deaminate L-valine or L-isoleucine besides L-alanine dehydrogenase.

Moreover, we tried Sephadex G-200 column chromatography to separate these enzymes. The samples that were obtained from the precipitate at 40 to 50% ammonium sulfate saturation with L-valine dehydrogenase and L-isoleucine dehydrogenase and that at 50 to

TABLE 2. *Purification and isolation of each dehydrogenase using* L-*alanine,* L-*valine, or* L-*isoleucine as substrates*

Enzyme fraction	Sp. act.[a]		
	L-Alanine[b]	L-Valine[b]	L-Isoleucine[b]
Crude enzyme preparation	75.0	31.6	34.2
Ammonium sulfate fractionation			
40–50%	20.1	123.0	148.6
50–70%	171.0	19.0	20.5
Sephadex G-200 column chromatography	475.0	500.0	527.0

[a] Values are expressed as nanomoles of reduced nicotinamide adenine dinucleotide per minute per milligram of protein.

[b] Substrate.

70% saturation with L-alanine dehydrogenase were respectively applied to a Sephadex G-200 column (1.5 by 20 cm; bed volume 35 ml) equilibrated with 50 mM phosphate buffer (pH 6.8). The results were shown in Fig. 1. In three dehydrogenases the enzyme activities gave the same positions in the elution patterns, and therefore L-valine dehydrogenase and L-isoleucine dehydrogenase could not be separated from L-alanine dehydrogenase by this method. Subsequently, an isoelectric fractionation by electrolysis was performed on the crude enzyme preparation obtained from the spores. As shown in Fig. 2, L-valine dehydrogenase and L-isoleucine dehydrogenase activities appeared at pH 2.2 and L-alanine dehydrogenase activity appeared at pH 4.5. However, the former deaminated L-valine, L-isoleucine, L-leucine, and slightly L-alanine, and the latter did not deaminate any other amino acid than L-alanine.

These results showed that there are two enzymes at least: one of them is L-alanine dehydrogenase which has substrate specificity, and the other is a dehydrogenase which is able to use L-valine, L-isoleucine, L-leucine, and L-alanine as substrates.

Comparison of various properties among L-alanine dehydrogenase, L-valine dehydrogenase, and L-isoleucine dehydrogenase. Other enzymatic properties were examined (Table 3). Molecular sizes of the three dehydrogenases were determined by gel filtration through a Sephadex G-200 column by using blue dextran 2,000 to determine the exclusion volume and catalase and bovine serum albumin to calibrate the column; the molecular weights were calculated by the method of Andrews (1). The approximate molecular weights of L-alanine, L-valine, and L-isoleucine dehydrogenases were respectively about 280,000, 290,000, and

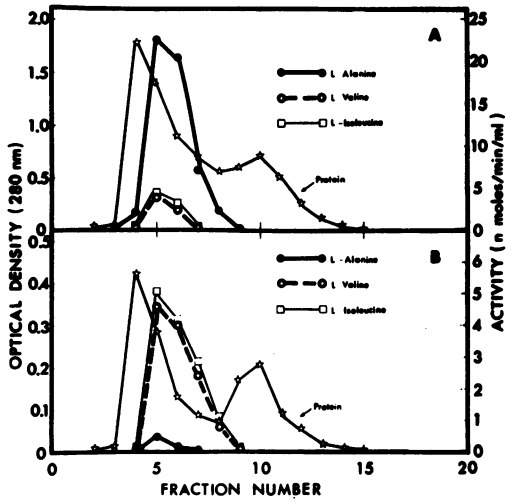

FIG. 1. *Elution patterns on Sephadex G-200 of* L-*alanine dehydrogenase,* L-*valine dehydrogenase, and* L-*isoleucine dehydrogenase in samples obtained from ammonium sulfate fractionation. Fractions of 5.0 ml each were collected. Activity of each enzyme was expressed as nanomoles of NAD reduced per minute per milliliter of each fraction, and protein was measured spectrophotometrically at 280 nm. A, 50 to 70% ammonium sulfate fractionation. B, 40 to 50% ammonium sulfate fractionation.*

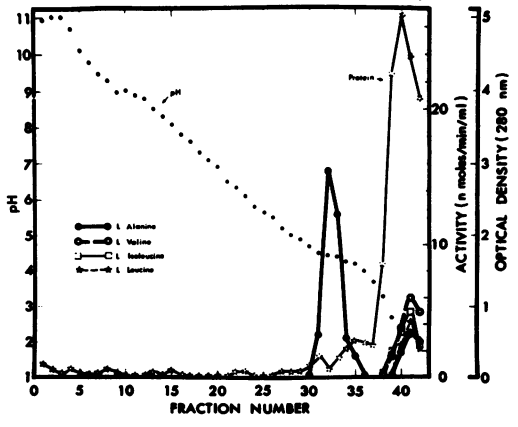

FIG. 2. *Isoelectric fractionation by electrolysis of crude enzyme preparation. Activities of* L-*alanine dehydrogenase,* L-*valine dehydrogenase,* L-*isoleucine dehydrogenase, and* L-*leucine dehydrogenase were expressed as nanomoles of NAD reduced per minute per milliliter of each fraction. Protein was measured spectrophotometrically at 280 nm.*

TABLE 3. *Comparison of enzymatic properties among* L-*alanine dehydrogenase,* L-*valine dehydrogenase, and* L-*isoleucine dehydrogenase*

Dehydrogenase	Approx mol wt[a]	Isoelectric point (pH)[b]	Optimum pH[b]	Heat stability[c] (remaining act, %)		K_m[a]		Effect of metallic[c] ions (remaining act., %)[e]			
				20 C[d]	50 C	Appropriate L-amino acid (M)	NAD (M)	Mg^{2+}	Ca^{2+}	Fe^{2+}	Zn^{2+}
L-Alanine	280,000	4.5	10.1	100	98	5.4×10^{-3}	3.6×10^{-4}	72	78	72	3
L-Valine	290,000	2.2	10.7	75	0	2.5×10^{-2}	3.3×10^{-3}	73	85	79	67
L-Isoleucine	290,000	2.2	10.7	65	0	2.2×10^{-3}	1.6×10^{-4}	94	95	84	86

[a] Ammonium sulfate precipitate.
[b] Crude enzyme.
[c] Sephadex fraction.
[d] Each enzyme solution was heated for 10 min.
[e] Final concentration of each metallic chloride was 3.3×10^{-3} M.

290,000. The optimal pH levels for the reactions catalyzed by these enzymes were 10.1 for L-alanine dehydrogenase and 10.7 for L-valine dehydrogenase and L-isoleucine dehydrogenase. Moreover, the heat stability of these enzymes was investigated. When these enzymes were incubated at 20 C for 10 min, 100% activity of L-alanine dehydrogenase, 75% of L-valine dehydrogenase, and 65% of L-isoleucine dehydrogenase remained. However, the activity of L-alanine dehydrogenase was scarcely lost for heating at 50 C for 10 min, whereas a total loss of L-valine dehydrogenase and L-isoleucine dehydrogenase occurred. The apparent K_m of L-alanine dehydrogenase calculated from the plot according to Lineweaver and Burk (8) were 5.4×10^{-3} M for L-alanine and 3.6×10^{-4} M for NAD. Those of L-valine dehydrogenase and L-isoleucine dehydrogenase were 2.5×10^{-2} M for L-valine and 2.2×10^{-3} M for L-isoleucine, and 3.3×10^{-2} M and 1.6×10^{-4} M for NAD, respectively. NAD in the oxidative deamination assay could not be replaced by NADP. We have further investigated the effects of various metallic ions on these enzymes; 3.3 mM Zn^{2+} inhibited 97% of L-alanine dehydrogenase, 33% of L-valine dehydrogenase, and 14% of L-isoleucine dehydrogenase.

Judging collectively from these results, L-valine dehydrogenase and L-isoleucine dehydrogenase seem to be the same enzyme and different from L-alanine dehydrogenase.

DISCUSSION

In this experiment it is shown that there are two kinds of L-amino acid dehydrogenase: one of them is the dehydrogenase using only L-alanine as a substrate, and the other is that using L-isoleucine, L-valine, L-leucine, or L-alanine. Optimum pH, K_m, heat stability, and molecular weight of the L-alanine dehydrogenase in our strain are almost the same with those reported by O'Connor and Halvorson (10). However, there is one important difference between the alanine dehydrogenase of *B. cereus* spores reported by them (11) and that of *B. subtilis* spores we used; the former is able to deaminate a number of L-amino acids other than L-alanine, but the latter only L-alanine. The discrepancy between them may be due to difference in species or to insufficient separation.

Since various properties of L-valine dehydrogenase and L-isoleucine dehydrogenase are almost similar except the K_m for NAD and the appropriate L-amino acid, both enzymes appear to be the same enzyme which has a broad spectrum of L-amino acids, and different from L-alanine dehydrogenase; we surmise, from K_m data, that this broad spectrum enzyme is probably L-isoleucine dehydrogenase. Sanwal and Zink (13, 17) obtained alanine dehydrogenase-free preparation of L-leucine dehydrogenase from vegetative cells of *B. cereus*, but not from its spores. Therefore, the data indicated here present the first report on the presence of an L-amino acid dehydrogenase other than L-alanine dehydrogenase in spores. The optimum pH, K_m for the appropriate substrate, and substrate specificity of L-isoleucine dehydrogenase of *B. subtilis* spores are very similar to those of L-leucine dehydrogenase of *B. cereus* cells, if the L-alanine dehydrogenase activity which appeared at pH 2.2 in the elution patterns by an isoelectric fractionation (Fig. 2) were due to a bimodal elution of the major L-alanine dehydrogenase; L-alanine is not active in oxidative deamination reaction by L-leucine dehydrogenase.

It is not clear whether the presence of L-isoleucine dehydrogenase in spores supports the pyruvate hypothesis (14). However, the two facts that (i) spores of alanine dehydrogenase negative mutants prepared in *B. subtilis* still are induced to germinate by L-alanine (4) and (ii) cysteine, methionine, serine, and glutamic acid support germination without a detectable corresponding dehydrogenase (Table 1) may not suggest the direct relationship between germination by L-amino acid and the corresponding dehydrogenase.

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