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PURIFICATION AND PROPERTIES OF L-ALANINE DEHYDROGENASE FROM VEGETATIVE CELLS OF *BACILLUS CEREUS*

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ABSTRACT

McCORMICK, NEIL G. (University of Wisconsin, Madison), AND HARLYN O. HALVORSON. Purification and properties of L-alanine dehydrogenase from vegetative cells of *Bacillus cereus*. J. Bacteriol. 87:68-74. 1964.—The L-alanine dehydrogenase from vegetative cells of *Bacillus cereus* strain T has been purified approximately 200-fold. The enzyme has a molecular weight of 248,000 and a turnover number of 80,000 moles of substrate per min per mole of enzyme. The Michaelis constants for the substrates and the equilibrium constant for the reaction catalyzed by this enzyme are in close agreement with reported values for other L-alanine dehydrogenases. The kinetic properties of the enzyme purified from vegetative cells are identical to those of the enzyme isolated from spores of the same organism, but differ with respect to relative heat stability. Whereas spores contain a heat-resistant enzyme, vegetative cells contain, in addition, a heat-sensitive enzyme. No evidence was found to support the hypothesis that a molecular conversion type of phenomenon plays a role in the appearance of spore enzyme.

The conversion of a vegetative cell to a dormant endospore involves changes in enzyme patterns and in the state of the enzymes. One of the key enzymes in the spore is L-alanine dehydrogenase, which is believed to function in the trigger mechanism for L-alanine–induced germination (Halvorson, O'Connor, and Doi, 1961). As has been reported (McCormick and Halvorson, 1963), the level of this enzyme in spores of *Bacillus cereus* strain T is enriched by sporulation in the presence of L-alanine.

Since the level of L-alanine dehydrogenase in spores, but not in vegetative cells, is increased by growth and sporulation in medium containing L-alanine, the question is raised as to the mechanism of this increase in enzyme content. Does the enzyme present in spores exist in a different form than that present in vegetative cells? Can a conversion hypothesis, analogous to the L-alanine dehydrogenase–L-glutamic dehydrogenase interconversions reported by Tomkins, Yielding, and Curran (1961), aid in explaining the changes in enzyme content in spores? The present paper describes the isolation and purification of L-alanine dehydrogenase from vegetative cells of *B. cereus* to test whether or not the conversion hypothesis might account for the observed changes in the level of L-alanine dehydrogenase.

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MATERIALS AND METHODS

Growth of cultures and preparation of cell-free extracts. Cells of *B. cereus* T were obtained by growth in a medium consisting of 1% Tryptone, 1% yeast extract, 1% glucose, and 0.5% K_2HPO_4 at pH 7.2. The cultures were incubated at 37 C for 16 to 18 hr and harvested in a Sharples centrifuge. The cell paste was resuspended in an equal volume of 0.01 M phosphate (pH 6.8) containing 0.01 M β-mercaptoethanol.

Cell-free extracts were obtained by passing a thick suspension of cells twice through a French pressure cell. The mixture was centrifuged at 27,000 $\times$ *g* for 20 min in a Servall centrifuge, and the resulting supernatant solution was used as a crude extract.

The methods for sporulation were those described by O'Connor and Halvorson (1960). Spore extracts were prepared by treatment in a Mini-Mill (Gifford-Wood Co., Hudson, N.Y.) in the presence of glass beads (100 μ in diameter).

Purification procedure. Crude extracts containing approximately 30 mg of protein per ml were treated by the addition of streptomycin sulfate to a final concentration of 1.5%. After 15 min, the mixture was centrifuged, and the supernatant fluid was further fractionated by the addition of varying amounts of solid ammonium sulfate. These precipitates were collected by centrifugation, dissolved in a minimal volume of 0.01 M phosphate buffer (pH 6.8) containing

0.01 M β-mercaptoethanol, and dialyzed overnight against several changes of the same buffer. The dialyzed preparation was added to the top of a diethylaminoethyl (DEAE)-cellulose column (1.5 by 25 cm) previously equilibrated with the above buffer. Chromatography was carried out by use of a linear gradient of phosphate buffer from 0.01 to 0.5 M (pH 6.8) in the presence of 0.01 M β-mercaptoethanol. Enzyme-containing fractions were pooled and concentrated by precipitation with ammonium sulfate followed by dialysis. The alkaline ammonium sulfate fractionation was accomplished by the addition of a saturated solution of ammonium sulfate previously adjusted to pH 8.0 with ammonium hydroxide. Best separations were obtained at this step when the extract was diluted to 1 to 2 mg of protein per ml with the phosphate-mercaptoethanol buffer.

Enzyme assays. L-Alanine dehydrogenase was measured spectrophotometrically at 340 mμ, with L-alanine and nicotinamide adenine dinucleotide (NAD) as substrates. In addition to the enzyme, the cuvettes contained 280 μmoles of carbonate buffer (pH 9.9), 40 μmoles of L-alanine, and 1 μmole of NAD, in a total volume of 2.0 ml. Units of enzyme activity are defined as the micromoles of reduced NAD (NADH) formed per minute. L-Glutamic dehydrogenase assays were performed as described by Tomkins et al. (1961).

Heat inactivation studies. Various dilutions of the purified L-alanine dehydrogenase (most commonly 5 to 50 μg/ml) were made in 0.01 or 0.05 M phosphate (pH 6.8) containing 0.01 M β-mercaptoethanol, and were placed in a water bath maintained at the desired temperature. The dilutions were made into preheated buffer. Zero time samples were taken immediately after the dilution was made. The samples were removed and added directly to cuvettes containing the complete assay mixture minus enzyme. This procedure was found to yield more reproducible results than did removal of samples and storage in an ice bath until the assay was performed.

RESULTS

Isolation and purification. The enzyme isolated from vegetative cells of *B. cereus* T has been purified approximately 200-fold, as described in Materials and Methods. The total activity of the preparation increased through the first two steps of purification (Table 1), probably due to the progressive removal of NADH oxidase. A similar observation was made by Bach and Sadoff (1962) during the purification of glucose dehydrogenase from *B. cereus*. The total units of activity originally present were arbitrarily taken to be the sum of those present in the ammonium sulfate fractions.

In the absence of β-mercaptoethanol, the enzyme activity declined rapidly, particularly in the more purified preparations. Some preparations which had lost more than half of their activity could be restored to 80 to 90% of the original activity by dialysis overnight in the presence of 0.01 M β-mercaptoethanol. L-Alanine dehydrogenases previously isolated from *B.*

TABLE 1. *Purification of L-alanine dehydrogenase from vegetative cells of Bacillus cereus T*

Enzyme fraction	Total protein	Total enzyme activity*	Specific activity†	Purification‡	Yield‡
	mg				
Crude extract	16,500	10,626	0.64	1.0	
Streptomycin sulfate treatment	13,500	12,075	0.99	1.2	
Ammonium sulfate fractionation					
0–50 %	6,000	966	0.17		
50–70 %	4,600	15,569	3.38	3.3	92
70–95 %	3,000	322	0.11		
DEAE-cellulose chromatography					
1st column	1,520	15,054	9.90	9.7	89
2nd column	481	14,490	30.11	29.5	86
Alkaline ammonium sulfate fractionation					
0–50%	14.0	290	2.11		
50–60%	26.4	5,184	196.4	192.5	31
60–65%	29.6	4,637	156.9	153.9	27
65–70%	13.9	451	32.8		

* Defined in Materials and Methods.

† Defined as micromoles of NADH per minute per milligram of protein.

‡ Purification and yield are based on the total enzyme initially present being equal to the sum of the activities present in the ammonium sulfate fractions.

subtilis (Piérard and Wiame, 1960), *Mycobacterium tuberculosis* (Goldman, 1959), and *B. cereus* spores (O'Connor and Halvorson, 1960) were found to be sulfhydryl enzymes. The enzyme reported here has also been found to be a sulfhydryl enzyme, and therefore the presence of a reducing agent might be expected to stabilize the enzyme. A preliminary investigation of the number of sulfhydryl groups per enzyme molecule resulted in a value of 6, which can only be taken as a minimal value at this time. Because of the stimulatory effect of β-mercaptoethanol, the reducing agent was routinely added during all stages of purification.

Physical properties. An ultracentrifugal analysis of the 50 to 60% saturated alkaline ammonium sulfate fraction was performed. A minor component consistently appeared in the pattern of each of several different purified preparations in which several additional fractionation procedures failed to result in a further increase in specific activity. The $S_{20,w}$ of the major component was calculated to be 10.2 Svedberg units. The same value was obtained using the sucrose gradient method of Martin and Ames (1961). An apparent diffusion constant of 3.8×10^{-7} cm^2 per sec was calculated from the ultracentrifuge data by the method of Schachman (1957). By using this value and assuming a partial specific volume, $\bar{V}$, equal to 0.75 ml/g, the molecular weight of the enzyme was estimated to be 248,000 g per mole.

If the preparation is taken to be approximately 65% pure (judged by the major component in the Schlieren pattern), then the specific activity of the pure enzyme would become 312 μmoles of NADH per min per mg of protein, and the turnover number calculated from this would be approximately 80,000 moles of NADH per min per mole of enzyme. That the latter is the more likely figure for the specific activity of the pure enzyme is borne out by the fact that specific activities of 300 to 325 have been observed on several occasions in the peak tubes from the second DEAE-cellulose column. The protein concentration in these tubes, however, was too low to permit an ultracentrifugal analysis.

L-Alanine dehydrogenase content of spores. The number of molecules of L-alanine dehydrogenase per spore was calculated by determining the number of spores before and after breakage, and by assuming that the enzyme was completely extracted from the broken spores. By taking the total enzyme activity as that exhibited by ammonium sulfate fractions (after removal of interfering NADH oxidase), and by using the value of 300 as the specific activity of the pure enzyme, the spores were estimated to contain approximately 14 molecules of L-alanine dehydrogenase per spore. A value of 12 was obtained by assuming that dry spores contain approximately 50% protein, and by using as the weight of a spore the experimentally determined value of 1.5×10^{-12} g. The alanine labeling experiments of Harrell and Halvorson (1955) had led them to conclude that the number of alanine binding sites per spore was greater than one.

Optimal pH. The optimal pH for the deamination reaction catalyzed by purified L-alanine dehydrogenase is shown in Fig. 1. The optimal pH for the purified enzyme is somewhat lower than that reported for the *B. subtilis* enzyme (Piérard and Wiame, 1960) and the enzyme from *B. cereus* spores (O'Connor and Halvorson, 1960). The difference may reflect the more purified state of the enzyme preparation reported here.

Kinetic properties. The Michaelis constants for each of the substrates of L-alanine dehydrogenase are shown in Table 2. The values are in close agreement with reported values for the enzymes

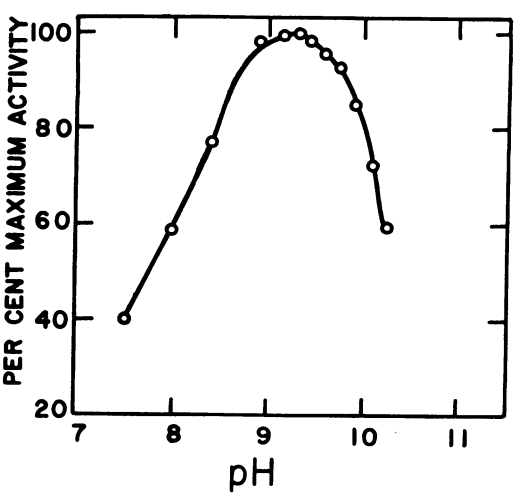

FIG. 1. *Effect of pH on the reaction catalyzed by purified L-alanine dehydrogenase. The enzyme assays were carried out as described in Materials and Methods in the presence of a mixture of tris(hydroxymethyl)aminomethane buffer and carbonate buffer, each adjusted to the desired pH. The pH values recorded are the final values at the termination of the experiment.*

TABLE 2. *Michaelis constants for substrates of L-alanine dehydrogenase**

Substrate	K_s
	M
L-Alanine	3.6×10^{-3}
NAD	4.8×10^{-5}
Pyruvate	5.3×10^{-4}
NH_4^+	1.5×10^{-2}
NADH	4.3×10^{-5}

* Deamination assays were carried out as described in Materials and Methods. For the amination assay, the reaction mixture contained, in addition to the enzyme, 700 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 9.0), 1.2 mmoles of ammonium chloride, 0.5 μmole of NADH, and 80 μmoles of sodium pyruvate, in a total volume of 2.0 ml.

isolated from *B. subtilis* (Piérard and Wiame, 1960), *B. cereus* spores (O'Connor and Halvorson, 1960), and *M. tuberculosis* (Goldman, 1959). The K_s for NH_4^+ does not agree too closely with that reported for *B. subtilis*, but is in good agreement with the other reported values. An equilibrium constant of $K_{eq} = 1.31 \times 10^{-14}$ corresponding to a $\Delta F = 18{,}850$ cal per mole is in accord with other values reported for the enzyme.

The purified preparation was completely inactive with respect to D-alanine and nicotinamide adenine dinucleotide phosphate (NADP). No activity could be demonstrated at any of several pH values with L-glutamate as substrate, even when the enzyme concentration was increased 100-fold. Traces of activity were detected with L-leucine as substrate.

Heat inactivation studies. The accumulated data suggest that the enzyme present in spores is identical to that present in vegetative cells. As a further test to establish the identity of the enzyme from the two sources, the heat stability of the enzyme preparations was determined. As shown in Fig. 2, the heat inactivation curve of the highly purified vegetative cell enzyme (curve B) is curvilinear. When the linear portion of curve B is extrapolated to zero time (curve C), it is seen that 81% of the initial enzyme activity is relatively heat-resistant, possessing a half-life of 80 min at 58 C. The derived heat inactivation curve of the remaining enzyme activity (curve D) results in a calculated half-life of 2 min for the heat-sensitive component. Curve A represents the heat inactivation curve of a partially purified L-alanine dehydrogenase preparation from *B. cereus* T spores. The observation of a linear heat inactivation curve for the spore enzyme is in agreement with results reported by O'Connor and Halvorson (1960). The calculated rate constant for the spore enzyme agrees well with the value for the heat-resistant component of purified vegetative cell enzyme (see Fig. 3).

An Arrhenius plot of the heat inactivation data is shown in Fig. 3. The data at each temperature were analyzed as described above and separated into two components. An apparent activation energy for heat inactivation of 17,400 cal per mole was determined for the heat-sensitive component. The major, heat-resistant component had a considerably higher activation energy of 142,000 cal per mole.

Cells were harvested at various stages during growth to determine whether the ratio of the two types of enzyme moieties changes during growth. Extracts were prepared, the enzyme preparations were taken through several steps of purification, and heat inactivation studies were carried out at 59 C. The heat inactivation curves were separated into two components as described for Fig. 2. Table 3 shows that the

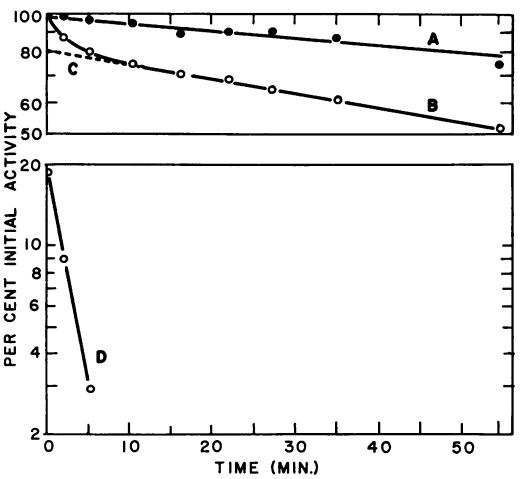

FIG. 2. *Heat inactivation of L-alanine dehydrogenase at 58 C. The experiment was conducted as described in Materials and Methods. Curve A = partially purified enzyme from spores. Curve B = highly purified enzyme from vegetative cells. Curve C = extrapolation for heat-resistant component of curve B. Curve D = extrapolation for heat-sensitive component of curve B (curve B — curve C).*

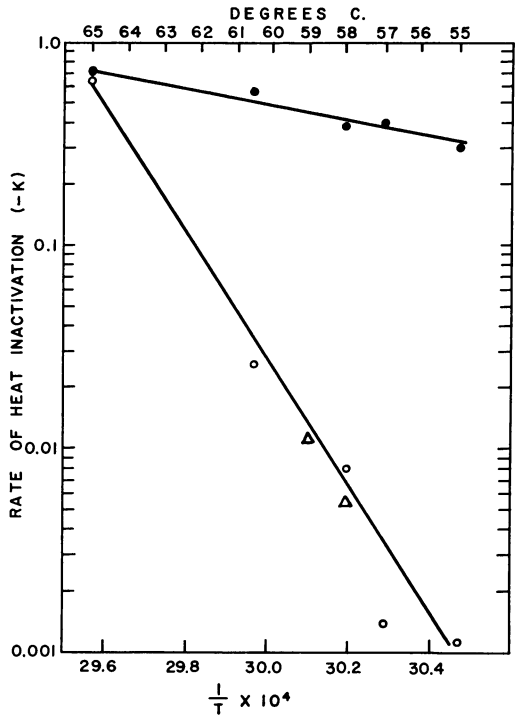

FIG. 3. *Arrhenius plot of heat inactivation of* L-*alanine dehydrogenase. Symbols:* ● = *heat-sensitive component of purified enzyme;* ○ = *heat-resistant component of the preparation;* △ = *partially purified spore enzyme.*

TABLE 3. *Variation in the levels of heat-resistant and heat-sensitive* L-*alanine dehydrogenase moieties as a function of the growth phase*

Growth phase	Per cent total activity*		Rate constant† $-k$	
	Resistant	Sensitive	Resistant	Sensitive
Middle log	33	67	0.0133	0.426
Early stationary	77	23	0.0118	0.414
Late stationary	91	9	0.0120	0.450
Spores	100	0	0.0110	—

* Determined as described in Fig. 2 and in Materials and Methods.

† Equal to 2.303 × (slope of heat inactivation curve of each component).

amount of the heat-resistant component (expressed as percentage of total activity) increases during growth. The inactivation rate constants calculated for the two components are in good agreement with the data in Fig. 3.

Attempts to convert L-*alanine dehydrogenase to* L-*glutamic dehydrogenase.* The enzyme pattern of spores can be influenced by the amino acid composition of the sporulation medium (McCormick and Halvorson, 1963). The level of L-alanine dehydrogenase in spores, but not in vegetative cells, is increased when L-alanine is present in the sporulation medium. The following possible explanations were considered: (i) a conversion phenomenon such as that described by Tomkins et al. (1961) on the reversible conversion between L-glutamic dehydrogenase and L-alanine dehydrogenase, (ii) a repression of vegetative-cell enzyme and an induction of spore enzyme during the sporulation process (*de novo* synthesis of spore enzyme), and (iii) an alanine (or alanine metabolite)-dependent transfer of vegetative-cell enzyme from the granular vegetative cell to the site of spore formation.

Tomkins et al. (1961) reported that, when crystalline beef liver glutamic dehydrogenase is dissociated to subunits by the action of certain steroid hormones, the subunit fraction possesses alanine dehydrogenase activity. This is apparently a reversible reaction and, upon reassociation, glutamic dehydrogenase activity is regained.

Treatment of purified *B. cereus* T L-alanine dehydrogenase under a variety of conditions which had been found to stimulate the association of subunits in the beef liver system caused varying levels of inhibition, but L-glutamic dehydrogenase activity could not be demonstrated. Attempts to influence the tertiary structure of the purified enzyme by mild treatment with urea followed by dialysis in the presence of glutamate and other compounds known to stimulate the association of subunits in the beef liver system were without effect. In only two instances could even a trace of glutamic dehydrogenase activity be demonstrated. These were with crude extracts of vegetative cells which were grown in a rich medium in the presence of 0.5% L-glutamate, and with spore extracts obtained from spores grown in sporulation medium to which L-threonine was added. Whether these two instances have any significance to the overall problem must await further experimentation. Thus, although L-glutamic dehydrogenase has been detected in *B. cereus* T and in other *Bacillus* species (Hong, Shen, and Braunstein, 1959), no evidence was found in

this study that L-alanine dehydrogenase is convertible into active L-glutamic dehydrogenase.

DISCUSSION

The origin of L-alanine dehydrogenase in spores is believed to arise at the onset of sporulation, either by an induction process or by the process of molecular conversion. The acquisition of a new catalytic property by a protein, which is dependent upon a reversible alteration in molecular structure, has been termed "molecular conversion" by Monod and Jacob (1961). The findings of Tomkins et al. (1961) that subunits (molecular weight, 2.5×10^5) of glutamic dehydrogenase (molecular weight, 10^6) possess L-alanine dehydrogenase activity and that, upon reassociation of the subunits, glutamic dehydrogenase activity is regained offers an attractive hypothesis for studying the evolution of L-alanine dehydrogenase in spores. Sanwal and Lata (1962) reported a "urea effect" in *Neurospora* which, during growth, represses the formation of a NADP-dependent glutamic dehydrogenase, and simultaneously induces the formation of a NAD-dependent glutamic dehydrogenase. They concluded that the molecular conversion hypothesis was not a likely explanation for their findings, and favored a repression-induction hypothesis.

Certain amino acids (L-leucine, L-methionine, L-isoleucine, and L-norvaline) have been found to stimulate the glutamic dehydrogenase reaction, whereas L-leucine prevented steroid influenced disaggregation to subunits (Yielding and Tomkins, 1961). If molecular conversions function in vivo, then one might expect that the addition of L-leucine to sporulating cultures of *Bacillus* would lead to a decrease in spore L-alanine dehydrogenase and a rise in the level of L-glutamic dehydrogenase. The results of such experiments indicated no essential difference in the level of alanine dehydrogenase; furthermore, no glutamic dehydrogenase activity could be detected. These results, together with the fact that no conversion could be detected with the purified alanine dehydrogenase, indicate that the molecular conversion mechanism probably does not play a role in the formation of spore L-alanine dehydrogenase. Either successful conversion experiments in the beef liver system involve some condition not yet realized in the *B. cereus* system, or the appearance of the enzyme in spores occurs by a *de novo* synthesis or by some other mechanism.

L-Alanine dehydrogenase purified from vegetative cells possesses two components, distinguishable by their different heat stabilities, but in all other respects is identical to the enzyme from spores. The ratio of the level of the heat-resistant enzyme to that of the heat-sensitive enzyme increases during growth. In the mature spore, only the resistant enzyme can be demonstrated. It has been pointed out that available evidence suggests that spore proteins and other unique spore components are derived from *de novo* synthesis during the sporulation process (Halvorson, 1962). The results reported above suggest that a regulatory mechanism operates during vegetative growth, resulting in a repression of the heat-sensitive L-alanine dehydrogenase and an induction in the synthesis of the heat-resistant enzyme.

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