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SEPARATION AND CHARACTERIZATION OF NAD- AND NADP-SPECIFIC SUCCINATE-SEMIALDEHYDE DEHYDROGENASE FROM *ESCHERICHIA COLI* K-12 3300

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Summary

Two distinct proteins endowed with succinate-semialdehyde dehydrogenase (succinate-semialdehyde:NAD(P)$^+$oxidoreductase, EC 1.2.1.16) activity were separated and partially purified by ammonium sulphate fractionation or Sephadex G-200 gel-filtration or both. They differ by coenzyme specificity (NAD or NADP), molecular weight, temperature and pH resistance, pH-activity curves, β-mercaptoethanol activation.

Moreover, the NADP-specific enzyme catalyzes only the oxidation of succinate-semialdehyde among a number of aldehydes tested, whereas the NAD-specific form is active also towards *n*-butyraldehyde. The K_m for the substrate is also appreciably different according to the coenzyme specificity, while the K_m values for NAD and NADP are quite similar.

Finally, the growth of the cells on γ-aminobutyrate as the sole source of nitrogen resulted in enhanced level of the NAD-dependent succinate-semialdehyde dehydrogenase, with concurrent decrease of the alternate enzyme activity.

On the basis of the above results, distinct metabolic roles are suggested for the two enzymes forms.

Introduction

It has been established that 4-aminobutyrate produced by glutamate decarboxylase (L-Glutamate 1-carboxy-lyase, EC 4.1.1.15) is converted to succinate through aminobutyrate-transaminase (EC 2.6.1.19) and succinate-semialdehyde dehydrogenase (NAD(P)$^+$) (EC 1.2.1.16) in nervous tissues [1—3] plants [4,5] and some microorganisms [6—8]. Among microorganisms,

Pseudomonas fluorescens, grown on pyrrolidine, was found to contain high levels of both enzymes, that could be purified to various extents [9—11]. Although it was reported that these enzymes are also present in wild-type strains of *Escherichia coli* K-12 and that their levels are increased in mutants growing on aminobutyrate [12], very limited data are so far available on the structural and functional properties of the *E. coli* enzymes. During the course of our studies on bacterial glutamate decarboxylase, we felt the need of a more exhaustive knowledge of the enzymes metabolizing the aminobutyrate. By checking the levels of such enzymes in microorganisms grown under different conditions, succinate-semialdehyde dehydrogenase activity was easily measurable in crude extracts of *E. coli* K-12 3300. Successive attempts to isolate and purify the enzyme led us to the separation of two protein fractions both having dehydrogenase activity, although endowed with different structural and functional properties and with distinct coenzyme specificity. Moreover, culture conditions were established, under which the NAD- and NADP-dependent activity levels were affected in opposite ways.

The present paper describes the methods of separation and some comparative properties of these two dehydrogenase forms, as well as some conditions controlling their activity levels in the living cells.

Materials and Methods

Chemicals. Succinate-semialdehyde, NAD and NADP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala, Sweden. The aldehydes checked as substrate of the dehydrogenase were obtained either from Aldrich Chemical Co., Milwaukee, WI, U.S.A., or from Sigma Chemical Co.

The standard enzymes employed for calibration of the Sephadex G-200 gel column were supplied from Boehringer, Mannheim, F.R.G. Other reagents and buffer salts were reagent grade materials.

Cell cultures, processing, and preparation of crude extracts. When not otherwise stated, they were the following: *E. coli* K-12 3300 from the collection of the Institut Pasteur (Paris) was grown on a mineral medium containing K_2HPO_4 $4 \cdot 10^{-2}$ M, KH_2PO_4 10^{-1} M, $(NH_4)_2SO_4$ $3 \cdot 10^{-2}$ M, $MgSO_4$ $8 \cdot 10^{-4}$ M, $FeSO_4$ $1.8 \cdot 10^{-6}$ M.

Glucose and other carbon and/or nitrogen sources were autoclaved separately and added aseptically to the mineral medium. The cells were grown overnight at 37°C, either in Erlenmayer flasks with vigorous shaking, or in standing Roux bottles, to provide aerobic or anaerobic conditions, respectively. The growth was followed by measuring the absorbance of the cell culture at 600 nm. Then the cells were harvested by centrifugation, washed once and suspended with 10 vols. of 0.1 M Tris-HCl buffer, pH 7.4, containing 1% β-mercaptoethanol. The cells were then broken by sonication for 3 min at 0°C in a MSE 100 W sonic disintegrator. The resulting extract was clarified by centrifugation at 15 000 $\times g$ for 15 min.

Enzyme assays. Dehydrogenase activity was estimated by measuring spectrophotometrically NADH or NADPH formation from NAD or NADP, using succinate-semialdehyde as substrate. The standard incubation mixture

contained in a total volume of 1 ml 1 mM NAD or NADP, 40 mM β-mercaptoethanol, 72 mM Tris-HCl, pH 9.0, and 300—15 μg of protein. After 5 min preincubation at 37°C, the reaction was started by addition of 0.5 mM succinate-semialdehyde. The reduction of pyridine coenzymes was followed at 340 nm in a Saitron 301 spectrophotometer. In the blank the substrate solution was replaced by an equal volume of water. To estimate the activities of the marker enzymes in the gel filtration experiments, the following assays were employed: Aspartate aminotransferase (L-Aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was measured spectrophotometrically, by coupling the transaminase reaction to the reduction of oxaloacetate in the presence of excess malate dehydrogenase (L-Malate:NAD^+ oxidoreductase, EC 1.1.1.37). Aldolase (D-Fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) activity was measured spectrophotometrically by coupling the reaction to the ancillary system triose-phosphate isomerase/glycerol phosphate dehydrogenase. Adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4) and catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) activities were determined from the absorbance decrease at 265 and at 240 nm, respectively.

Protein estimation. The protein concentration of the enzyme preparations was determined by the method of Lowry et al. [13].

Gel filtration. It was performed an a Sephadex G-200 gel column (2 × 34 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 1% β-mercaptoethanol. Elution was carried out with the same buffer at a flow rate of 18 ml per h, by collecting 2 ml fractions.

Results

Separation of NAD- and NADP-dependent dehydrogenase. The separation was achieved both by $(NH_4)_2SO_4$ fractionation and by Sephadex gel-filtration. Table I summarizes the results obtained by both methods, which are described as following:

(1) Ammonium sulphate fractionation: The crude extract, catalyzing the

TABLE I

SEPARATION OF NAD- AND NADP-DEPENDENT SUCCINATE-SEMIALDEHYDE DEHYDROGENASE FROM *ESCHERICHIA COLI* K-12 3300

Dehydrogenase units are expressed as nmol NADH or NADPH formed per min, under standard assay conditions, as reported in the text.

Step	Volume (ml)	Total protein (mg)	NAD-dependent		NADP-dependent	
			total activity	specific activity	total activity	specific activity
Crude extract	24.5	228	2326	10.2	5517	24.2
48% $(NH_4)_2SO_4$ precipitation	26	53	2300	43.4	5368	101
63% $(NH_4)_2SO_4$ precipitation	2.5	18.2	2106	116	263	14.5
95% $(NH_4)_2SO_4$ precipitation	2.5	26.5	511	19	5240	198
Gel filtration:						
NAD-dependent fraction	5.2	1.7	1845	1090	147	87
NADP-dependent fraction	5.2	1.9	348	183	4445	2340

oxidation of succinate-semialdehyde both with NAD and with NADP, was brought to 48% saturation with solid $(NH_4)_2SO_4$ and the resulting precipitate, devoid of dehydrogenase activity, was discarded by centrifugation. Additional $(NH_4)_2SO_4$ was added to the supernatant to bring the saturation to 63%. The resulting precipitate, collected by centrifugation, contained the NAD-dependent dehydrogenase activity. Finally the $(NH_4)_2SO_4$ concentration of the supernatant was raised to 95% saturation. By centrifugation a precipitate was collected that contained the NADP-dependent dehydrogenase activity.

(2) Sephadex G-200 gel-filtration. The fractions containing the NAD- or NADP-specific dehydrogenase obtained by the above procedure were either separately submitted to Sephadex G-200 gel-filtration or they were combined before gel-filtration through the same column, obtaining superimposable elution patterns of dehydrogenase activities. As shown in Fig. 1, the mixture of the two coenzyme specific dehydrogenase fractions obtained by $(NH_4)_2SO_4$ fractionation is resolved in two distinct peaks, identical to those obtained by separate elution of the single fractions (not reported in figure). In this latter instance we also failed to detect dehydrogenase activity at the expected position for the alternative enzyme form in the elution diagram.

Molecular weight. By calibration of the Sephadex G-200 gel column with marker enzymes (Fig. 1), the molecular weights corresponding to the NAD- and NADP-specific dehydrogenase were determined. From the semilog plot (Fig. 1, inset) molecular weights of 96 000 and 120 000 were calculated for the NAD- and NADP-specific dehydrogenase, respectively.

Effects of temperature and pH. Table II summarizes the comparative effects of storage at different temperatures on the NAD- and NADP-dependent dehydrogenase. It can be observed that the NADP-dependent dehydrogenase is more sensitive to temperature rises than the NAD-dependent enzyme. Storage

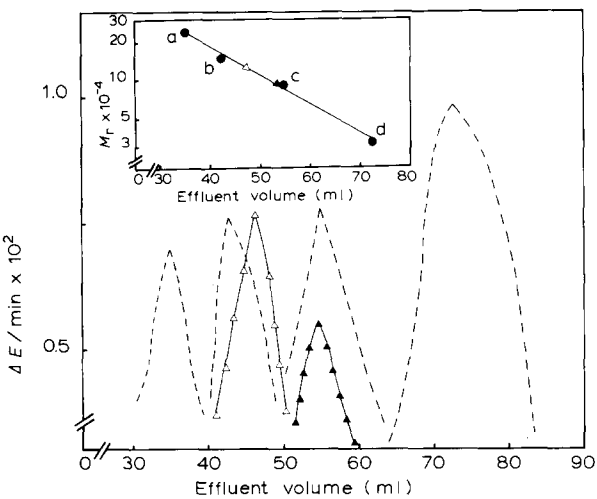


Fig. 1. Separation and molecular weight determination of NAD and NADP specific dehydrogenase by gel-filtration. Comparative profiles (main tracing) and comparative molecular weights (inset) of the NAD- (▲) and NADP- (△) specific dehydrogenase and of marker enzymes (- - - - -, ●): a, beef liver catalase, M_r 240 000 [17]; b, rabbit muscle aldolase, 158 000 [18]; c, pig heart aspartate amino transferase, 90 000 [19]; d, calf intestine adenosine deaminase, 33 500 [20].

TABLE II

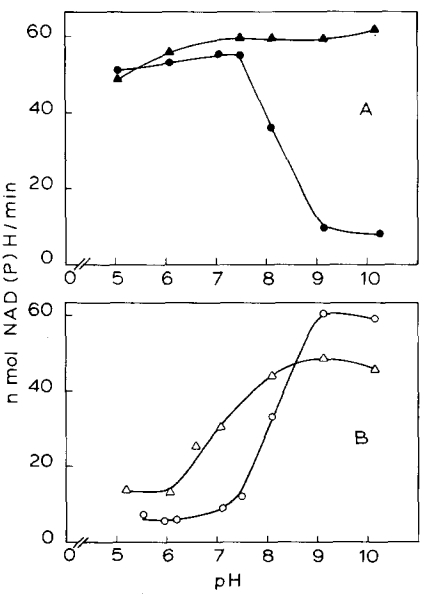
COMPARATIVE THERMOSTABILITY OF NAD- AND NADP-DEPENDENT SUCCINATE-SEMIALDEHYDE DEHYDROGENASE FROM *ESCHERICHIA COLI* K-12 3300

Aliquots of each coenzyme specific preparation were stored for 14 h at the temperatures indicated, before assay under standard conditions.

Temperature (°C)	NAD-dependent dehydrogenase residual activity (% of initial)	NADP-dependent dehydrogenase residual activity (% of initial)
−15	95	81
4	95	84
18	93	78
25	93	70
37	92	59

for different times gave consistent results with those reported in the table. Freezing and thawing resulted in complete inactivation of the NADP-dependent enzyme. The effect of pH in the range 5—10.1 was studied on both stability and catalytic activity (Fig. 2 A and B respectively) of NADP- and NAD-dependent dehydrogenase. The latter enzyme is stable on the whole pH range, while the NADP-dependent enzyme is sensitive to rises of pH to alkaline values.

With respect to catalytic activity, both enzymes display optimum pH around nine; however, the decrease of activity with the lowering of the pH to neutral and acidic values is much sharper with the NADP-dependent enzyme than with the NAD-specific dehydrogenase.



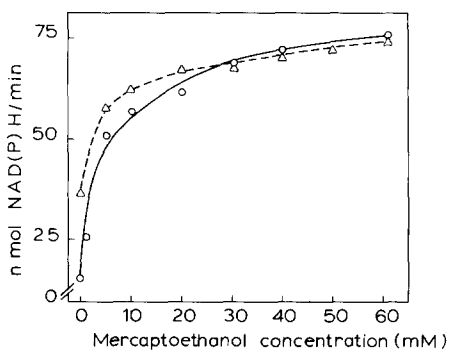


Fig. 2. Comparative curves of stability (A) and catalytic activity (B) of NAD- (△, ▲) and NADP- (○, ●) specific dehydrogenase as function of pH.

Fig. 3. Comparative activation of NAD- (△) and NADP- (○) specific dehydrogenase by β-mercaptoethanol. The reported thiol concentrations were added to a preparation of enzyme dialyzed 24 h (with one buffer change) against 5l of deionized water adjusted to pH 7 with Tris-HCl.

Activation by β-mercaptoethanol. The comparative effects of increasing concentrations of β-mercaptoethanol on NAD- and NADP-specific preparations of dehydrogenase are reported in Fig. 3. After exaustive dialysis, the NADP enzyme loses about 80% of original activity, whereas the NAD enzyme still retains almost 50% of maximum activity. Addition of β-mercaptoethanol up to 10—20 mM increases the reaction velocity of both dehydrogenase preparations, but the NAD enzyme becomes saturated earlier than the alternate enzyme. Higher thiol concentrations result in only slight activation. For practical purposes, 40 mM concentrations were chosen for the standard incubations.

Substrate specificity. Both the NAD- and the NADP-dependent dehydrogenase from *E. coli* display high substrate specificity. The following aldehydes were not attacked at 1 mM concentration under the same conditions where the true substrate is oxidized by both enzymes at high rate: formaldehyde, acetaldehyde, glyoxal, glyoxalate, propionaldehyde, glutaraldehyde, benzaldehyde, and anisaldehyde. An important difference, however, was observed with *n*-butyraldehyde; this is attacked at an appreciable rate by the NAD-dependent enzyme, the initial reaction velocity being 11% of that observed with succinate-semialdehyde (1 mM; standard assay conditions). In contrast no reaction was detected with the NADP-specific dehydrogenase. The possible significance of this observation will be discussed later.

K_m values for substrate and coenzymes. The K_m values for the specific pyridine coenzyme as well as those for the substrate were determined with each dehydrogenase preparation. The experimental data, reported in Figs. 4 and 5, show that the affinity of the NAD-dependent enzyme for the substrate (K_m $4.68 \cdot 10^{-5}$ M) is significantly lower than that of the NADP-dependent dehydrogenase (K_m for succinate semialdehyde: $1.01 \cdot 10^{-5}$ M). In contrast the K_m values for the pyridine coenzymes are quite similar ($3.75 \cdot 10^{-5}$ M and $4.02 \cdot 10^{-5}$ M for NAD and NADP, respectively).

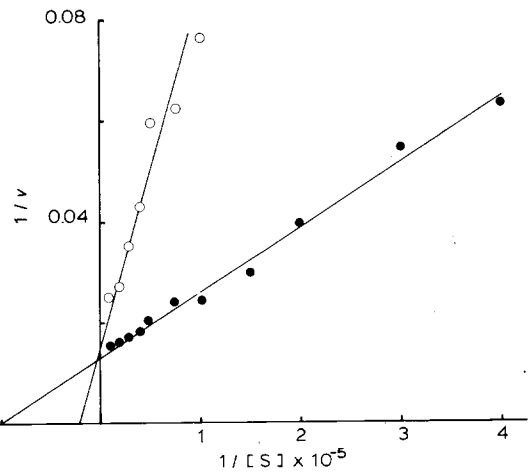


Fig. 4. Lineweaver-Burk plots of NAD- (○) and NADP- (●) specific dehydrogenase for the substrate. The Lineweaver-Burk plots were obtained by measuring the initial reaction velocities (v: nmol NADH or NADPH formed per min) at the concentrations of succinate-semialdehyde [S] indicated, with saturating coenzyme (1 mM NAD or NADP).

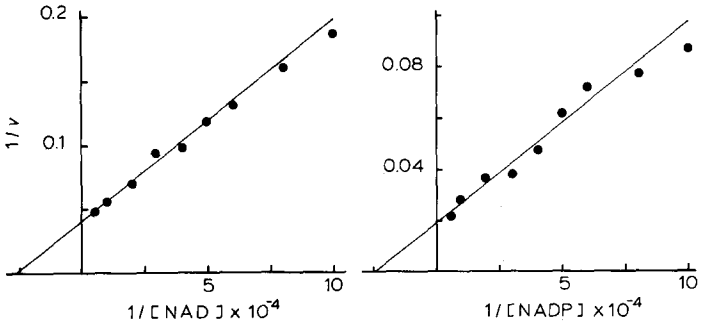


Fig. 5. Linveweaver-Burk plots of NAD- and NADP-specific dehydrogenase for the coenzyme. The double-reciprocal plots were obtained by measuring the initial reaction velocities (v: nmol NADH or NADPH formed per min) at the coenzyme concentrations indicated, with saturating substrate (0.5 mM succinate-semialdehyde).

NAD- and NADP-dependent dehydrogenase activities in cells grown under different culture conditions. The levels of dehydrogenase activities, using NAD and NADP as coenzymes, were checked in crude extracts of cells grown under different culture conditions, as reported in Table III. It was observed that the source of nitrogen or carbon and the oxygen availability, although affecting largely the generation times, did not considerably change the specific dehydrogenase activities with either coenzyme, except in the case when 4-aminobutyrate was used as sole source of nitrogen. In this instance, as a consequence of the reduction of the NADP-dependent dehydrogenase and concurrent increase of the alternate enzyme, the ratio of specific activities NAD-

TABLE III

NAD- AND NADP-DEPENDENT SUCCINATE-SEMIALDEHYDE DEHYDROGENASE ACTIVITIES IN *ESCHERICHIA COLI* K-12 3300 GROWN UNDER DIFFERENT CULTURE CONDITIONS

The cells were either grown in the complete mineral medium (reported under Methods), supplemented with the indicated source(s) of carbon (C) (0.5%); or in the same medium without $(NH_4)_2SO_4$, completed by the test compound (0.5%), with or without glucose to check the ability of the compound to support the growth as a nitrogen (N) source or as source of N and C, respectively. The growth was tested under aerobic conditions, except in the case indicated. The generation times were determined from semilog plots of absorbance at 600 nm vs. time. Dehydrogenase specific activities were determined in crude cell extracts and expressed as nmol NADH of NADPH formed per min per mg protein. n.d., not determined.

Growth conditions	Generation time (min)	NADP dehydrogenase sp. act.	NAD dehydrogenase sp. act.
Glucose	50	24.2	10.2
Glucose (anaerobic)	120	22.3	9.3
Glycerol	40	20.4	8.8
Glutamate (C) + glucose	50	23.7	9.8
Glutamate (C)	120	19.5	7.9
Glutamate (N) + glucose	300	21.6	9.0
Glutamate (C + N)	>600	n.d.	n.d.
Aminobutyrate (C) + glucose	50	23.4	10.8
Aminobutyrate (C)	>600	n.d.	n.d.
Aminobutyrate (N) + glucose	360	9.6	27.1

dehydrogenase/NADP-dehydrogenase rose up to 2.8 (from the fairly constant value of approx. 0.4, observed in the other conditions).

Discussion

The experimental data reported above strongly indicate that in *E. coli* K-12 3300 the oxidation of succinate-semialdehyde is catalyzed by two distinct proteins, differing both in their structural features (molecular weight, electric charges) and in their functional properties (specificity of coenzyme, sensitivity to temperature and pH, pH-activity curve, activation by β-mercaptoethanol, K_m for succinate semialdehyde). This conclusion is further supported by the comparative levels of NAD- and NADP-dependent dehydrogenase activities in cells grown on 4-aminobutyrate as sole source of nitrogen (Table III). This result clearly indicates that the two enzyme forms can undergo in vivo distinct and even opposite regulations.

By the purification procedure reported above (Table I) the specific activity of both enzymes is increased about 100-fold with respect to crude extracts. However, both the NAD- and the NADP-specific fractions still display dehydrogenase activity with the alternate coenzyme. This result seems more likely due to inherent enzyme activity rather than to incomplete fractionation, as suggested by the ratios of activities with NAD and NADP at the various separation steps (Table I) and by the results of the gel filtration experiments.

The presence of both NAD- and NADP-specific dehydrogenase was reported in some *Pseudomonas* species [14]. Although the contemporary isolation and the comparative properties of the two enzymes from the same *Pseudomonas* strain were not reported, it is interesting that the NAD-specific enzyme possessed low inherent aminoaldehyde dehydrogenase activity, attacking 4-aminobutanal and 3-aminopropanal [15]. Our data on substrate specificity suggest that a similar condition could be present in *E. coli* 3300.

The possible physiologic role of the two coenzyme specific dehydrogenases is only a matter of speculation. It seems unlikely that a differential regulation of the two enzyme forms present in *E. coli* can be obtained through the control of the coenzyme concentrations, as suggested for the enzymes of *Agaricus bisporus* [16], since in our case both dehydrogenases have high and very similar affinities for NAD and NADP. In contrast, the different K_m for the substrate could suggest the involvement of the two enzymes in distinct metabolic routes. In fact, the higher affinity of the NADP-dependent enzyme for the substrate could allow a preferential draining of the succinate-semialdehyde into some anaplerotic path whenever the concentration of substrate is low. But when the substrate level rises, much of it could be used up as fuel, by entering the Krebs' cycle as succinate through the NAD-dependent dehydrogenase. This assumption received some support by our recent observation (unpublished) that the NAD-dependent enzyme is more sensitive to specific inhibitors, e.g. oxaloacetate and phospho*enol*pyruvate, than the NADP-dependent enzyme. Further support to this hypothesis comes from the above reported results on the in vivo regulation. When ammonia is supplied solely by 4-aminobutyrate, the production of succinate-semialdehyde is possibly enhanced as a consequence of the increased rate of the aminobutyrate-transaminase reaction. Hence the need of

increase of the 'catabolic' NAD-dependent dehydrogenase and that of more strict control of the alternate dehydrogenase, possibly producing NADPH for anaplerotic or biosynthetic reactions.

Better understanding of the physiologic significance of the coenzyme-specific dehydrogenases of *E. coli* will probably be provided by the current investigation on their molecular and regulatory properties, as well as on their location in the bacterial cell.

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