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Properties and synthesis regulation of NAD(P)H dehydrogenases from *Rhodobacter capsulatus*

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Three NAD(P)H dehydrogenases were found and purified from a soluble fraction of cells of the purple non-sulfur bacterium *Rhodobacter capsulatus*, strain B10. Molecular mass of NAD(P)H, NADPH and NADH dehydrogenases are 67000 (4 · 18000), 35000 and 39000, and the isoelectric points are 4.6, 4.3 and 4.5, respectively. NAD(P)H dehydrogenase is characterized by a higher sensitivity to quinacrine, NADPH dehydrogenase by its sensitivity to *p*-chloromercuribenzoate and NADH dehydrogenase by its sensitivity to sodium arsenite. In contrast to the other two enzymes, NAD(P)H dehydrogenase is capable of oxidizing NADPH as well as NADH, but the ratio of their oxidation rates depends on the pH. All NAD(P)H dehydrogenases reacted with ferricyanide, 2,6-dichlorophenolindophenol, benzoquinone and naphthoquinone, but did not exhibit transhydrogenase, reductase or oxidase activity. Moreover, NADH dehydrogenase was also capable of reducing FAD and FMN. NAD(P)H and NADH dehydrogenases possessed cytochrome-*c* reductase activity, which was stimulated by menadione and ubiquinone Q_1. The activity of NAD(P)H and NADH dehydrogenases depended on culture-growth conditions. The activity of NAD(P)H dehydrogenase from cells grown under chemoheterotrophic aerobic conditions was the lowest and it increased notably under photoheterotrophic anaerobic conditions upon lactate or malate growth limitation. The activity of NADH dehydrogenase was higher from the cells grown under photoheterotrophic anaerobic conditions upon nitrate growth limitation and under chemoheterotrophic aerobic conditions. NADPH dehydrogenase synthesis dependence on *R. capsulatus* growth conditions was insignificant.

Introduction

NAD(P)H dehydrogenases (= reductases) are important components of electron transport chains of chemotrophic and phototrophic organisms. Homogeneous preparations of these enzymes were obtained from *Bacillus subtilis* [1], *Escherichia coli* [2], *Thermus aquaticus* [3], *Rhodobacter sphaeroides* [4], *Rhodobacter capsulatus* $37b_4$ [5,6], *Thiocapsa roseopersicina* [7,8] and *Nostoc* sp. [9]. However, the functions of NAD(P)H dehydrogenases in photosynthesis, hydrogen and nitrogen metabolism, as well as their synthesis regulation, have not been adequately studied.

The available data on synthesis regulation of NAD(P)H dehydrogenases from phototrophic bacteria mainly concern respiratory chain NADH dehydrogenase. Here, we show that NADH dehy-

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate; TNTC, tetranitro blue tetrazolium chloride; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

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drogenase activity of *R. capsulatus* Kbl, *Rhodopseudomonas palustris* and *Rhodospirillum tenue* cells increased during their aerobic cultivation in the dark [10–12]. However, under such growth conditions no changes of NADH dehydrogenase activity were found in *Rhodospirillum rubrum* and *R. capsulatus* while NADH oxidase activity increased [13,14]. It has been suggested [14] that the cytochrome *c* oxidase segment of the respiratory chain is responsible for the overall increase in respiratory chain activity [14].

Several NAD(P)H dehydrogenases which differ in location and properties are found in *R. rubrum* [13,15–17], *T. roseopersicina* [7,8], *Rps. palustris* [18] and *R. capsulatus* $37b_4$ [5]. However, in earlier studies concerning the effect of growth conditions on the activity of NAD(P)H dehydrogenases, the presence of various forms of this enzyme, performing, probably, various functions was not taken into account.

The isolation and study of properties as well as of synthesis regulation and the possible functions of NAD(P)H dehydrogenases from purple nonsulfur bacterium *R. capsulatus* B10 was the aim of the present study.

Materials and Methods

Culture conditions

R. capsulatus B10 cells were grown in Ormerod medium [19] with thiamine in a device for continuous cultivation of phototrophic microorganisms [20]. Chemoheterotrophic aerobic or photoheterotrophic anaerobic growth in turbidostat and chemostat upon organic compound (lactate, malate) or nitrogen source (ammonium, nitrate) limitations were carried out as described earlier [21–23]. Gas mixture (200 ml/min), containing 2% CO_2 + 98% Ar [21] was passed through a fermenter during culture growth under anaerobic photoheterotrophic conditions. In some experiments, the gas mixture was passed through a tube containing a copper catalyst heated to 300 °C and through a solution of methyl viologen, reduced by metallic zinc with an activated surface to eliminate traces of oxygen.

Preparation of cell extracts and fractions

To prepare cell extracts and fractions, fresh or thawed cells (20–60 g) were suspended (1 : 3) in 20 mM potassium-phosphate buffer (pH 6.5) (which was used throughout this work, unless otherwise stated) and disrupted by sonication (22 kHz; 0.4 A; 4–10 °C). Whole cells and debris were removed by centrifugation (20000 × *g* for 30 min at 4 °C). Soluble and membrane fractions were obtained after high-speed centrifugation of the crude extract (144000 × *g* for 120 min at 4 °C).

Preparation of orange agar gel

Agar gel (6%) desulfated and cross-linked by epichlorohydrine was prepared according to Porath [24]. It was dyed by the method described in Ref. 25, using active bright-orange triazine dye KCh. The amount of bound dye was determined by the method described in Ref. 26. 1 ml of the orange agar gel contained 1.2–1.8 mg of the dye.

Analytical methods

Relative molecular weights of NAD(P)H dehydrogenases were estimated by gel filtration [27] on a Sephadex G-150 column (1.1 × 60 cm), preequilibrated with a 150-mM solution of NaCl. The molecular weight and subunit composition of NAD(P)H dehydrogenase were also determined by SDS-electrophoresis according to Weber and Osborn [28]. Cytochromes *c* from horse and swine heart (M_r 12500 and 12400, respectively), aldolase (40000), ovalbumin (43000), bovine serum albumin (68000), chymotrypsinogen (25700) and blue dextran (2000000) were used as markers.

The isoelectric points of the enzymes were determined by the micro method of vertical isoelectrofocusing in columns (0.5 × 8.0 cm) of polyacrylamide gel within the pH range 3–10 and 3.5–5.0 [29]. Electrophoresis was carried out in 7% gel in Tris-glycine buffer (pH 8.3) [30].

NAD(P)H dehydrogenase activity was assayed spectrophotometrically [31]. The reaction mixture contained 0.25 mM NAD(P)H, 1 mM ferricyanide, 0.01 mM FMN and 50 mM potassium-phosphate buffer (pH 6.5) up to 1 ml. The reaction was initiated by addition of the enzyme preparation (0.1–2 units) to the reaction mixture.

NAD(P) reductase activity was assayed by oxidation of benzyl viologen reduced by dithionite in the presence of NAD(P) [32], and NAD(P)

reduction by membrane cell fraction, according to Ref. 33.

Transhydrogenase activity was assayed by NADP reduction in the presence of NADH-generating system and by NAD reduction in the presence of NADPH-generating system [32]. Cytochrome *c* reductase activity was measured by an absorbance change at 552 nm [33]. Oxidase activity was determined spectrophotometrically by NADH oxidation of the air. The reduction of ferredoxins was monitored by the change of their absorbance spectrum. All reactions except the reduction of NAD(P) and ferredoxins were carried out at 20 °C under aerobic conditions.

The lactate concentration in the medium was assayed by the NAD reduction reaction with lactate dehydrogenase [35] and the amount of protein, according to Lowry [36] and Bradford [37], using bovine serum albumin as the standard.

Chemicals

DCIP and TNTC were purchased from Chemapol, Czechoslovakia; α-chymotrypsinogen, NAD(P)H, DEAE-cellulose and reagents for electrophoresis were from Reanal, Hungary; Swine heart cytochrome *c* was from Biomed-Krakov, Poland; SDS was from Serva, F.R.G.; FMN was from Merck, F.R.G.; G- and R-250 Coomassie and blue dextran were from Ferak, West Berlin; bovine serum albumin was from Boehringer, F.R.G.; Sephadex G-75 and G-100, 5′-AMP-Sepharose and ampholytes were from Pharmacia, Sweden, menadione was from BDH Ltd., U.K.; quinacrine was from Sigma, U.S.A. and agar was from Difco, U.S.A. Other materials were of the highest purity commercially available.

Results

Localization and forms

Crude extracts of *R. capsulatus* B10 cells exhibited both NAD(P)H and NADH dehydrogenase activities. After sonication of cells or their treatment with lysozyme, the greater part of NAD(P)H dehydrogenase activities was found in a soluble fraction. There were about 2.6–3.7% of NADPH and 6.2–8.7% of NADH dehydrogenase activity in the membrane fraction.

During soluble fraction chromatography on a column (4 × 10 cm) with DEAE-cellulose, two fractions were observed (Table I). Fraction 1 was eluted with 150 mM NaCl in a buffer and exhibited both NADPH and NADH dehydrogenase activities, the second being greater in this case. Fraction 2 was eluted with 250 mM NaCl and exhibited mainly NADPH dehydrogenase activity (Table I).

About 5% of NADH dehydrogenase activity did not bind (fraction 3) with orange gel (2 × 7 cm), while adding fraction 1 preliminarily concentrated and diluted 3-fold (to decrease NaCl concentration). Up to 80% of the enzyme activity was eluted by 500 mM NaCl in buffer (pH 8.0) (fraction 4). NADPH and NADH dehydrogenase activities were similar in this fraction (Table I).

During subsequent chromatography on columns with orange gel and DEAE-cellulose, fraction 2 did not divide into several forms. Thus, from *R. capsulatus* cells, three fractions were obtained with NAD(P)H dehydrogenase activities: fraction 2 interacted mainly with NADPH (NADPH dehydrogenase), fraction 3 with NADH (NADH dehydrogenase) and fraction 4 both with

TABLE I

NAD(P)H dehydrogenases in a soluble fraction of *R. capsulatus*

Purification steps	Activity with NADH		Activity with NADPH	
	units	$K^{FMN}_{stimulus}$ [a]	units	$K^{FMN}_{stimulus}$
Soluble fraction	525.0	1.9	550.0	1.4
DEAE-cellulose column chromatography:				
Fraction 1	350.8	2.5	304.0	1.6
Fraction 2 (NADPH dehydrogenase)	35.3	1.9	84.6	1.1
Orange-gel column chromatography [b]:				
Fraction 3 (NADH dehydrogenase)	47.9	1.3	15.3	1.7
Fraction 4 (NAD(P)H dehydrogenase)	162.9	8.0	169.3	9.0

[a] Degree of stimulation of activity in the presence of FMN.
[b] Fraction 1 was used for the purification.

NADPH and NADH (NAD(P)H dehydrogenase). The activity of the latter depended on FMN.

NAD(P)H dehydrogenase purification

A soluble fraction of *R. capsulatus* B10 cells was applied to a column (4 × 10 cm) with DEAE-cellulose DE-52. The column was washed with a buffer and the enzyme was eluted with 150 mM NaCl. During this process, the spec. act. of the enzyme increased 3-fold with 80–96% of the activity being retained (Table II). The fraction obtained was concentrated by ultrafiltration through a membrane 'Ripor-4', then diluted 3-fold and applied to a column with orange agar gel. The column was then washed with buffers of pH 6.0 and 8.0. The enzyme was eluted by 500 mM NaCl in pH 8.0 buffer.

Orange gel binding capacity for NAD(P)H dehydrogenase was 250–500 units of activity per 1 ml of gel. 40–80% of the enzyme was bound with gel (by activity) or 0.5–1.5% (by protein), providing a 30 to 100-fold purification of NAD(P)H dehydrogenase.

NAD(P)H dehydrogenase binding was not affected by the presence of FMN (0.04 mM) in the enzyme preparation and buffer, equilibrating the column and FMN solution (0.04 and 1 mM) did not elute the adsorbed enzyme [38]. Active bright-orange dye (0.015 mM) suppressed the enzyme activity by 81%. FMN (0.01 mM) had some protective effect, but in the presence of 2 mM NADH, the degree of NAD(P)H dehydrogenase inhibition was considerably decreased. The enzyme inhibition by the dye appeared to be competitive vs. NADH ($K_i = 4\ \mu M$) [38].

The preparation eluted from the column with orange gel was concentrated by the method described above and further enzyme purification was carried out by preparative electrophoresis. After this stage of purification, the spec. act. of NAD(P)H dehydrogenase increased to 5000 units/mg, the total degree of purification was 1000 to 1700-fold, the output was 10% by activity and 0.006% by protein (Table II). During repeated electrophoresis, the enzyme migrated as a single band with an R_f value of 0.68, proving its electrophoretic homogeneity.

NADPH dehydrogenase purification

Fraction 2 eluted with 250 mM NaCl from a column (4 × 10 cm) with DEAE-cellulose (Table I) was used for NADPH dehydrogenase purification. The fraction was 3-fold diluted with a buffer and applied to a column (1 × 5 cm) with DEAE-cellulose DE-52 (Table III) and equilibrated with 20 mM potassium-phosphate buffer (pH 7.5). The column was washed with the same buffer with a decreasing pH value (7.5 → 5.4 → 5.2). The enzyme was eluted by 75 mM potassium-phosphate buffer (pH 5.0), while the majority of protein was desorbed with 200 mM KH_2PO_4. At this stage, the specific enzyme activity increased 8 to 13-fold (Table III).

The preparation obtained was concentrated twice, diluted 5-fold in order to decrease ionic strength and then applied to a column (1 × 12 cm) with orange gel. In contrast to NAD(P)H dehydrogenase, NADPH dehydrogenase binding to the orange gel was weaker. The enzyme absorption was obtained only at low pH values (5.2) or on a column equilibrated with a distilled water and while using preliminarily purified preparation. The enzyme was eluted from the column with 500 mM NaCl.

TABLE II

PURIFICATION SCHEME OF NAD(P)H DEHYDROGENASE FROM *R. capsulatus*

Purification steps	Protein (mg)	Total act. (units)	Spec. act. (units/mg protein)	Purification (fold)	Yield (%)
Soluble fraction	1330	3857	2.9	1	100.0
DEAE-cellulose column chromatography (elution by 150 mM NaCl)	444	3130	7.05	2.4	81.2
Orange-gel column chromatography (elution by 500 mM NaCl)	2.2	1800	800	276	46.7
Preparative electrophoresis	0.08	385	5000	1724	10.0

TABLE III

PURIFICATION SCHEME OF NADPH DEHYDROGENASE FROM *R. capsulatus*

Purification steps	Protein (mg)	Total act. (units)	Spec. act. (units/mg protein)	Purification (fold)	Yield (%)
DEAE-cellulose column chromatography (elution by 250 mM NaCl)	25	37.5	1.5	1	100
DEAE-cellulose column rechromatography (elution at pH 5.0)	1.2	15.0	12.5	8	40
Orange-gel column chromatography (elution by 500 nM NaCl)	0.2	11.0	56	37	29
5′-AMP-Sepharose	0.03	3.7	125	83	9.8

Bright-orange dye inhibited the NADPH dehydrogenase activity, while NADPH decreased the extent of inhibition. In this case a mixed type of inhibition was observed, also, the K_i was about 100 μM, which is considerably higher than K_i for NAD(P)H dehydrogenase. The spec. act. of the enzyme at this stage increased 2 to 5-fold.

The preparation obtained was concentrated twice, diluted 10-fold and applied to a column (1.2 × 3.5 cm) with a 5′-AMP-Sepharose equilibrated by 20 mM potassium-phosphate buffer (pH 6.5). The enzyme was weakly bound with a sorbent and eluted without changing the buffer. Due to some retardation of NADPH-dehydrogenase as compared with the other proteins a 2-fold enzyme purification was achieved. The obtained preparation was electrophoretically homogeneous (Fig. 1) and its spec. act. was 125 units/mg (Table III).

NADH dehydrogenase purification

Fraction 3 which did not absorb on a column with orange gel (Table I) was concentrated and applied to a column (1.1 × 60 cm) with Sephadex G-100 ('medium') and equilibrated with 150 mM NaCl in pH 6.6 buffer. Fractions with NADH dehydrogenase activity were pooled, concentrated and used as a partially purified preparation of NADH dehydrogenase. Its spec. act. was 11 units/mg.

Properties of NAD(P)H dehydrogenases

Relative molecular weights of NAD(P)H, NADPH and NADH dehydrogenases assayed by gel filtration were 67000, 35000 and 39000, respectively. During electrophoresis of the first enzyme in the presence of SDS, a single protein band with M_r 18000 was found (Fig. 1). This indicates that the enzyme consists of four identical subunits.

NAD(P)H, NADPH and NADH dehydrogenases from *R. capsulatus* B10 have similar isoelectric points (4.6, 4.3 and 4.5, respectively).

The activity of diluted NAD(P)H dehydrogenase preparations (0.01 mg/ml) decreased 9-fold during 24 h (20 ° C) storage. The addition of FMN (0.01 mM) and, to a lesser extent, of glycerol (25%) stabilized the enzyme. The concentrated

Fig. 1. SDS-gels of NAD(P)H dehydrogenase (1) and NADPH dehydrogenase (2).

enzyme preparations were more stable. During long-term storage (−4°C), their stability increased in the presence of glycerol and, to a lesser extent, of bovine serum albumin. NADPH and NADH dehydrogenases appeared to be more stable [31].

In contrast to NAD(P)H dehydrogenase, the activity of NADPH and NADH dehydrogenases did not depend on FMN. Quinacrine (0.2 mM), interacting with flavoproteins, suppressed the activity of NAD(P)H and NADH dehydrogenases by 57% and 36%, respectively (Table IV). However, it did not affect NADPH dehydrogenase, the activity of which was suppressed by 0.2 mM PCMB (by 84%). PCMB also had an inhibitory effect on the activity of NAD(P)H dehydrogenase (by 47%). Partially purified NADH dehydrogenase was more sensitive to 1 mM sodium arsenite (by 54%) (Table IV).

Incubation with NADH partially protected NAD(P)H dehydrogenases from an PCMB inhibitory effect. NADPH and NADH had an activating effect on NADH dehydrogenase, whereas the activity of NADPH dehydrogenase after its preincubation with NADPH (0.5 mM) was decreased by 45% (Table IV). All three enzymes exhibited a weak sensitivity to iodoacetate and chelating compounds (α,α'-dipyridyl, *o*-phenanthroline, EDTA).

Catalytic properties

NADPH dehydrogenase oxidized only NADPH ($V_{NADPH} = 97$ units/mg, $V_{NADH} = 0$), NADH dehydrogenase oxidized mainly NADH ($V_{NADH} \approx 11.6$ units/mg, $V_{NADPH} \approx 1$ units/mg). NAD(P)H dehydrogenase reacted both with NADPH and NADH ($V_{NADPH} = V_{NADH} = 6600$ units/mg).

This enzyme exhibited higher NADPH dehydrogenase activity at pH < 6.5 but higher NADH dehydrogenase activity at pH > 7 (Fig. 2). At pH ≈ 6.5 oxidation of these compounds proceeded with similar rates. In contrast to NAD(P)H dehydrogenase, at pH < 6 NADH dehydrogenase is more active than NADPH dehydrogenase.

All three NAD(P)H dehydrogenases from *R. capsulatus* B10 reduced ferricyanide, DCIP, benzoquinone and naphthoquinone, but differed by their relative rates of reduction (Table V). NADH dehydrogenase and, to a lesser extent, NAD(P)H dehydrogenase reduced ubiquinone Q_1, but not the other ubiquinones. NADH and NAD(P)H dehydrogenases were found to possess cytochrome *c* reductase activity stimulated by menadione and ubiquinone Q_1 (Table V). NADH dehydrogenase also exhibited the ability of FMN and FAD reduction under aerobic as well as anaerobic conditions. However, none of the three NAD(P)H dehydrogenases of *R. capsulatus* B10 catalyzed

TABLE IV

EFFECT OF SOME COMPOUNDS ON THE ACTIVITIES OF NAD(P)H DEHYDROGENASES

Enzyme activity was determined in the presence of DCIP (0.04 mM) after 15 min incubation of the preparations. n.d., not determined

Compounds	Concentration (mM)	Activity suppression (%)		
		NAD(P)H dehydrogenase	NADPH dehydrogenase	NADH dehydrogenase
Iodoacetate	1.0	18	n.d.	10
PCMB	0.002	n.d.	76	n.d.
PCMB	0.2	47	84	9
Rotenone	0.01	+8 [a]	0	0
o-Phenanthroline	1.0	27	n.d.	n.d.
α,α'-Dipyridyl	1.0	11	8	10
EDTA	1.0	27	0	+3
Quinacrine	0.2	57 [b]	+9	36
Dicoumarol	0.1	25	5	15
Sodium arsenite	1.0	19	6	54
NADPH	0.5	0	45	+15
NADH	0.5	n.d.	+6	+23

[a] Activity stimulation (+).

[b] Activity was determined in the absence of FMN both in the experimental and control variants.

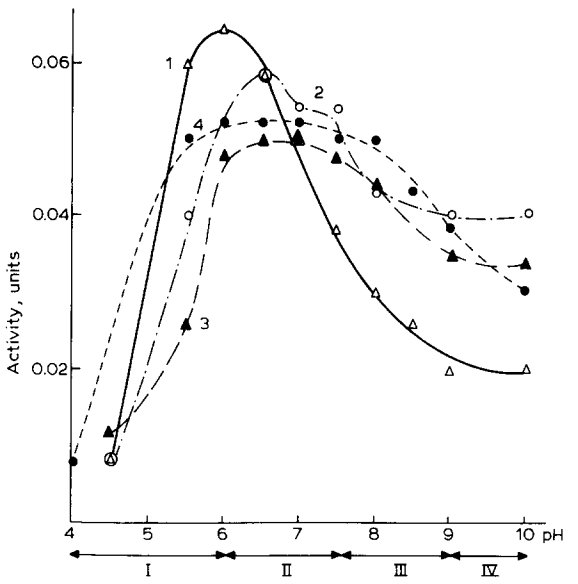

Fig. 2. Dependence of the activities of NAD(P)H dehydrogenases from *R. capsulatus* on the pH value of the reaction mixture: 1 (△), NAD(P)H dehydrogenase (with NADPH); 2 (○), NAD(P)H dehydrogenase (with NADH); (3) (▲), NADPH dehydrogenase; 4 (●), NADH dehydrogenase. I, acetate buffer; II, sodium-potassium-phosphate buffer; III, Tris-HCl buffer; IV, Glycine buffer. Experimental conditions for determination of the dehydrogenase activities are shown in Table I. FMN (0.01 mM) was added while determining NADPH and NADH dehydrogenase activity of NAD(P)H dehydrogenase.

NAD(P) reduction in the presence of reduced benzyl viologen, as well as the reduction of ferredoxins, benzyl and methyl viologens. Neither did they exhibit any transhydrogenase or oxidase activity.

NAD(P)H dehydrogenase had equal K_m values for NADPH and NADH (76 μM). They are considerably higher than those for NADPH and NADH dehydrogenases ($K_m^{NADPH} = 30$ μM and $K_m^{NADH} = 10$ μM). NAD(P)H, NADPH and NADH dehydrogenases slightly differ in their K_m values for ferricyanide (54, 83 and 80 μM, respectively).

Effect of cultivation conditions on the activity of NAD(P)H dehydrogenases

Our preliminary observations were made on total NADPH and NADH dehydrogenase activities in *R. capsulatus* B10 cells extracts and did not take into account the existence of three forms of NAD(P)H dehydrogenases.

The total NADPH and total NADH dehydrogenase activities were maximal in *R. capsulatus* B10 cells, grown under photoheterotrophic anaerobic conditions in a chemostat under organic compound limitation (Table VI). Under such conditions an increase of the activities correlated with a decrese of the flow rate and lactate concentration in the medium. The change of these activities during lactate-limited growth is unlikely to be

TABLE V

NAD(P)H OXIDATION RATES IN THE PRESENCE OF VARIOUS ELECTRON ACCEPTORS

Enzyme activity with ferricyanide of the same concentration as that of other acceptors is assumed to be 100%.

Electron acceptor	Concentration (mM)	NAD(P)H dehydrogenase	NADPH dehydrogenase	NADH dehydrogenase
DCIP	0.017	69	25	18
2,6-Dimethoxy-1,4-benzoquinone	0.017	58	0	282
Naphthoquinone	0.17	100	35	200
Ubiquinone Q_1	0.017	38	traces	57
1,4-Benzoquinone	0.5	90	31	277
Menadione	0.5	30	traces	22
FMN	0.5	0	0	63 (60) [a]
FAD	0.5	0	0	57
Cytochrome *c*	0.05	traces	0	2.5
Cytochrome *c* + ubiquinone Q_1 (0.01 mM)	0.05	3	traces	19 (15)
Cytochrome *c* + menadione (0.01 mM)	0.05	12 (14) [a]	4.2	34 (26) [a]

[a] Under anaerobic conditions.

TABLE VI

NAD(P)H DEHYDROGENASE ACTIVITIES IN *R. capsulatus* CELLS GROWN UNDER VARIOUS CONDITIONS AND THEIR DISTRIBUTION ON A COLUMN WITH ORANGE GEL

The reaction mixture contained 0.5 mM NAD(P)H, 1 mM ferricyanide, 0.01 mM FMN, 50 mM potassium phosphate buffer (pH 6.5). The activity is presented in μmol ferricyanide/min·mg protein^{-1} (units/mg). The reaction was monitored at 420 nm.

Growth conditions	Spec. act. (units/mg protein)		Activity (%) [a]			
			fraction 1 [b]		fraction 2 [c]	
	NADH	NADPH	NADH	NADPH	NADH	NADPH
Photoheterotrophic, anaerobic:						
Turbidostat, lactate + NH_4^+	1.1	1.1	48	47	45	45
Chemostat under limitation of:						
Lactate	2.4	2.3	20	13	71	80
Malate	2.5	2.4	19	18	69	60
NH_4^+	1.1	0.9	47	37	35	38
NO_3^-	2.8	1.4	58	13	19	15
Chemoheterotrophic, aerobic:						
Turbidostat, lactate + NH_4^+	1.4	0.7	70	32	11	11
Chemostat under NH_4^+ limitation	1.2	0.4	81	36	16	13

[a] NADH dehydrogenase activity of the initial fraction, applied to a column (25 or 250 units in 15 ml) is assumed to be 100%.
[b] The activity of the fraction not adsorbed on the orange gel.
[c] The activity of the fraction eluted by 500 mM NaCl.

related to the O_2 effect, since after preliminary purification of argon from oxygen traces, the activities did not alter. Under such conditions no change of NADH oxidase activity was observed, although during the growth under aerobic conditions in the dark this activity was 5 to 8-fold higher.

Under photoheterotrophic anaerobic conditions during NO_3^--limited growth the total NADH dehydrogenase activity of cells increased. NADPH dehydrogenase activity did not essentially differ from that of cells grown under NH_4^+ limitation. During growth in a turbidostat, the replacement of NH_4^+ by glutamate resulted only in a negligible change of NaD(P)H dehydrogenase activity. During *R. capsulatus* B10 growth under chemoheterotrophic aerobic conditions, NADPH dehydrogenase activity decreased, whereas NADH dehydrogenase activity of cells remained at the same level (Table VI).

Using columns with orange gel, a fraction containing mainly NAD(P)H dehydrogenase and a fraction containing NADPH and NADH dehydrogenases were obtained (Table VI). Measuring the activities in these fractions, the contribution of the three enzymes to total NAD(P)H dehydrogenase activity of cells and the spec. act. of each enzyme were calculated.

From the data obtained it can be seen (Table VI) that in *R. capsulatus* B10 cells grown under photoheterotrophic anaerobic conditions during lactate- or malate-limited growth, the greater part of NAD(P)H dehydrogenase activity (60–80%) was due to FMN-dependent NAD(P)H dehydrogenase. The content of this enzyme (by activity) was minimal (11–13%) during chemoheterotrophic aerobic growth (Table VI). The content of NADH dehydrogenase increased during cell growth on NO_3^- medium under photoheterotrophic anaerobic conditions or under aerobic conditions in the dark (Table VI).

From the data obtained it is also seen (Table VII) that the most considerable changes in spec. act. under various growth conditions are characteristic of NAD(P)H dehydrogenase. The activity of this enzyme was minimal in cells grown in the dark under aerobic conditions (0.2 units/mg) and increased during growth in the light (0.5 units/mg) and especially under lactate- or malate-limited growth (1.9 units/mg). The activ-

TABLE VII

SPECIFIC ACTIVITY (units/mg) OF NAD(P)H DEHYDROGENASES DEPENDING ON *R. capsulatus* CELL GROWTH CONDITIONS

Growth conditions	NAD(P)H dehydrogenase	NADPH dehydrogenase	NADH dehydrogenase
Photoheterotrophic, anaerobic:			
Turbidostat, lactate + NH_4^+	0.5	0.6	0.6
Chemostat under limitation of:			
Lactate	1.9	0.3	0.5
Malate	1.9	0.5	0.5
Malate	1.9	0.5	0.5
NH_4^+	0.5	0.4	0.6
NO_3^-	0.7	0.7	2.1
Chemoheterotrophic, aerobic:			
Turbidostat, lactate + NH_4^+	0.2	0.5	0.9
Chemostat under NH_4^+ limitation	0.2	0.3	1.0

ity of NADH dehydrogenase increased not only during culture growth under photoheterotrophic anaerobic conditions on NO_3^- medium, but also under aerobic conditions in the dark (Table VII). In contrast to these enzymes NADPH dehydrogenase did not exhibit notable activity dependence on *R. capsulatus* B10 growth conditions (Table VII).

Discussion

Soluble, as well as membrane-bound NAD(P)H dehydrogenases were found in purple bacteria *Rh. rubrum, Rps. palustris* and *R. capsulatus* [5,10,13,15–18]. However, only in the case of *R. capsulatus* $37b_4$ it has been shown that soluble and membrane-bound NADH dehydrogenases differ in a number of properties (Table VIII). NADPH dehydrogenase, the properties of which have not been studied yet, is also found in a soluble fraction of this bacterium [5]. Three NADPH dehydrogenases are now found in *R. capsulatus* B10, but, in contrast to strain $37b_4$ [5], all of them are detected in a soluble fraction and one of them, NAD(P)H dehydrogenase, is nonspecific to pyridinenucleotides (Table VIII). It is possible that the absence of the latter enzyme in *R. capsulatus* $37b_4$ was due to the fact that the cells of this bacterium were grown under aerobic conditions in the dark [6]. Under such conditions according to our data (Table VII), the content of this enzyme in *R. capsulatus* B10 cells was insignificant.

The enzymes from *R. capsulatus* strains B10 and $37b_4$ differ considerably both in a set of NAD(P)H dehydrogenases and their properties (Table VIII). NAD(P)H dehydrogenase from *R. capsulatus* B10 is probably a flavoprotein, since its activity depends on FMN, which also has a stabilizing effect during storage of diluted preparations, while quinacrine suppresses the activity of this enzyme. The fact that flavin is easily separated from NAD(P)H dehydrogenase upon dilution, chromatography and other stages of purification indicates that it is bound with apoprotein via a non-covalent bond. The activity is restored upon addition of FMN but not FAD or riboflavin to the reaction mixture. NADH dehydrogenases, containing weakly bound flavins were also found in *Rh. rubrum* and *R. capsulatus* $37b_4$ [6,15]. In contrast to NAD(P)H dehydrogenase, NADPH and NADH dehydrogenases from *R. capsulatus* B10 are not activated by flavins, although quinacrine suppresses the activity of the latter enzyme.

NAD(P)H dehydrogenases of *R. capsulatus* B10 differ one from another not only in a number of physico-chemical properties, but also in donor-acceptor specificity and sensitivity to inhibitors (Tables IV, V and VII). Only FMN-dependent NAD(P)H dehydrogenase can interact both with NADPH and NADH. The ratio of their oxidation rates depends on the medium pH (Fig. 2). The change of NAD(P)H dehydrogenase affinity to an electron donor with the medium pH is probably the way of its activity regulation, which is important for the function of this enzyme in vivo. As well as NAD(P)H dehydrogenases of phototrophic bacteria, other enzymes are also known which show a relative specificity to pyridine nucleotides. In these cases, the rates and Michaelis constants for NADPH and NADH-dependent reactions are different [39,40].

The particular property of NAD(P)H dehydro-

TABLE VIII

PROPERTIES OF NAD(P)H DEHYDROGENASES FROM DIFFERENT *R. capsulatus* STRAINS

Properties	Strain B10			Strain $37b_4$ [5,6]	
	NAD(P)H dehydrogenase	NADPH dehydrogenase	NADH dehydrogenase	NADH dehydrogenase	NADH dehydrogenase
Localization	cytoplasm	cytoplasm	cytoplasm	membranes	cytoplasm
M_r	67000	35000	39000	97000	37000
p*I*	4.6	4.3	4.5	n.d.	n.d.
pH optimum	5.5–6.4 [a] 6.2–7.2 [b]	6.0–7.5	5.5–8.0	7–9	7–8.5
FMN effect	stimulated	not stimulated	not stimulated	inactivated	stimulated
Activity (%):					
With ferricyanide	100	100	100	181	176
With DCIP	19	15	50	100	100
With 1,4-benzoquinone	90	31	27	n.d.	n.d.
With cytochrome *c*	12 [c]	4 [c]	34 [c]	2.8	150
$K_m^{NAD(P)H}$ (mM)	0.076 [a] 0.076 [b]	0.03	0.01	0.004	0.045
$K_m^{acceptor}$ (mM)	0.054 [d]	0.083 [d]	0.07 [d]	0.063 [e]	0.0079 [e]
Inhibition (%)					
Quinacrine	57	+9	36	n.d.	n.d.
PCMB	47	84	9	n.d.	n.d.
Arsenite	19	6	54	+32	n.d.
NADPH	0	45	+15	8	n.d.
NADH	15	+6	+23	95	n.d.

[a] Activity with NADPH.
[b] Activity with NADH.
[c] In the presence of menadione.
[d] Ferricyanide as an acceptor.
[e] DCIP as an acceptor.

genase from *R. capsulatus* B10 is its high catalytic activity, reaching 5000 μmol/min · mg protein^{-1} in the NAD(P)H oxidation reaction, whereas the activity of similar enzymes from other purple bacteria does not exceed 100 μmol/min · mg protein^{-1} [5,7,8,40].

The three NAD(P)H dehydrogenases of *R. capsulatus* B10 also differ in their ability to interact with electron acceptors (Table VIII). Only NADH dehydrogenase reduces FMN and FAD at a relatively high rate (Table V). It resembles NADH dehydrogenase *R. capsulatus* AD2 in this property [41], but, in contrast to the latter, the reaction proceeds under anaerobic as well as aerobic conditions.

Similar to NAD(P)H dehydrogenases (= reductases) from *R. rubrum, Rb. sphaeroides, R. capsulatus* $37b_4$, *T. roseopersicina* and *Chlorobium limicola forma thiosulfatophilum* [4,6–8,15,40], NADH dehydrogenase and, to a lesser extent, NAD(P)H dehydrogenase from *R. capsulatus* B10 catalyze the cytochrome *c* reduction reaction. However, in contrast to NADP reductases from *Bacillus polymyxa* [34] and spinach [42,43], cytochrome reduction by these enzymes was stimulated by menadione and ubiquinone Q_I (Table V), but not by ferredoxin.

All three NAD(P)H dehydrogenases of *R. capsulatus* B10 exhibited no transhydrogenase or oxidase activities. Neither did they catalyze the reactions with low potential electron carriers (ferredoxin, benzyl and methyl viologen). By this property, the described enzymes differ from NAD(P) reductase of *Rps. palustris* [44], *Ch. limicola f. thiosulfatophilum* [40] and *T. roseopersicina* [7,8].

There is no available data concerning the effect of growth conditions on the synthesis of various NAD(P)H dehydrogenase forms and their ratios in the cells of phototrophic bacteria. According to our data, with respect to *R. capsulatus* B10, the activity of two enzymes (NAD(P)H and NADH dehydrogenase) can vary 4 to 10-fold and their ratio, nearly 20-fold (Table VII). The lowest NAD(P)H dehydrogenase activity was observed in cells grown under chemoheterotrophic aerobic conditions. It indicates that this enzyme does not participate in the respiratory chain. NAD(P)H dehydrogenase activity was notably higher in cells grown under photoheterotrophic anaerobic conditions especially during limitation of organic compounds (Table VII). The latter is probably due to derepression of enzyme synthesis [45]. Under such growth conditions the activity of hydrogen uptake by hydrogenase in *R. capsulatus* B10 increases considerably due to its derepression [46]. Therefore, it cannot be excluded that the function of NAD(P)H dehydrogenase of *R. capsulatus* B10 can be connected with NAD(P) reduction, e.g., due to hydrogen. In fact, a membrane fraction obtained from cells exhibited the ability to reduce NAD(P) in the presence of hydrogen or succiante. The rate of NAD(P) reduction by molecular hydrogen in the presence of an ATP-generating system was comparable to the rate of succinate-dependent reduction of NAD (21–29 nmol NAD(P)H/h). Analogous data were obtained earlier for *R. capsulatus* Kbl [33], grown in batch cultures under photoautotrophic conditions. Stimulation of NAD(P) reduction with a membrane fraction of *R. capsulatus,* strain Kbl [33], or B10 in the light or in the presence of ATP in the dark indicates that its occurrence is probably due to energy-dependent reverse-electron transfer.

There is little data concerning the functions of NADP-specific dehydrogenases from phototrophic bacteria. NADPH-dependent enzymes from some purple bacteria as well as ferredoxin-NADP reductases from cyanobacteria and higher plants are suggsted to take part in NADP photoreduction [39,40]. It has also been shown that NADPH dehydrogenase from *Escherichia coli* is related to the sulfite-reductase complex [47]. In cyanobacteria, NADPH as well as NADH dehydrogenases are probably components of the respiratory chain [48,49]. We found no considerable changes in the activity of NADPH dehydrogenase from *R. capsulatus* B10 under various growth conditions, indicative of its function in the cells.

The observed increase of NADH dehydrogenase activity (Table II) indicates that this enzyme participates not only in respiration but in nitrate reduction as well. According to the data recently obtained in our laboratory, a membrane fraction of *R. capsulatus* B10 cells grown in medium with NO_3^-, contains a nitrate reductase of a dissimilar type [50]. Similar to *R. capsulatus* BK5 nitrate reductase [41], it is able to use NADH as one of electron donors, providing the reduction of nitrates.

Under chemoheterotrophic aerobic conditions of *R. capsulatus* B10 growth, an increase of NADH dehydrogenase activity could be revealed only after preliminary separation of NAD(P)H dehydrogenases, owing to a simultaneous decrease of the activity of NAD(P)H dehydrogenase, which also using NADH as an electron donor. In this connection it cannot be excluded that a similar cause may account for contradictions of earlier data concerning the effect of chemoheterotrophic aerobic growth on NADH dehydrogenase activity of purple bacteria *R. rubrum, Rh. tenue, R. capsulatus* and *Rb. palustris* [10,11,13,51,52].

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