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AN NAD(P) REDUCTASE DERIVED FROM *CHLOROBIUM THIOSULFATOPHILUM*: PURIFICATION AND SOME PROPERTIES

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SUMMARY

A highly purified preparation of an NAD(P) reductase was obtained from *Chlorobium thiosulfatophilum* and some of its properties were studied. The enzyme possesses FAD as the prosthetic group, and reduces benzyl viologen, 2,6-dichlorophenolindophenol and cytochromes *c*, including cytochrome *c*-555 (*C. thiosulfatophilum*), with NADPH or NADH as the electron donor. It reduces $NADP^+$ or NAD^+ photosynthetically with spinach chloroplasts in the presence of added spinach ferredoxin. It reduces the pyridine nucleotides with reduced benzyl viologen. The enzyme also shows a pyridine nucleotide transhydrogenase activity. In these reactions, the type of pyridine nucleotide (NADP or NAD) which functions more efficiently with the enzyme varies with the concentration of the nucleotide used; at concentrations lower than approx. 1.0 mM, NADPH (or $NADP^+$) is better electron donor (or acceptor), while NADH (or NAD^+) is a better electron donor (or acceptor) at concentrations higher than approx. 1.0 mM. Reduction of dyes or cytochromes *c* catalysed by the enzyme is strongly inhibited by $NADP^+$, 2′-AMP and atebrin.

INTRODUCTION

In green plant photosynthesis, it is known that photoreduction of $NADP^+$ requires the cooperation of a flavoprotein (NADP reductase) and a non-haem iron protein (ferredoxin)[1,2]. The same mechanisms have been found for $NADP^+$ reduction in algae such as *Anabaena variabilis*[3] and *Navicula pelliculosa*[4].

A similar enzymatic system seems to function in bacterial photosynthesis. Thus, an NADP reductase and a non-haem iron protein have been isolated from *Rhodopseudomonas palustris*, and it has been shown that they are required for the photoreduction of $NAD(P)^+$ by an acid-treated particulate preparation which contains bound bacteriochlorophylls[5]. Although, in this experiment, $NADP^+$ is more rapidly reduced under appropriate conditions, NAD^+ is also reduced at an appreciable rate. This is very different from the case of the green plants. From these facts, it might be expected that in more primitive photosynthetic organisms the pyridine nucleotide reductase functions only with NAD^+. It seems interesting

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

to know whether the specificity of the reductase for pyridine nucleotide is related to the evolutionary degree of the organism from which the enzyme is isolated.

Chlorobium thiosulfatophilum is thought, on physiological[6] and biochemical[7] grounds, to be very close to the most primitive photosynthetic organism. We have obtained a highly purified preparation of pyridine nucleotide reductase from this green sulphur bacterium. This enzyme shows various enzymatic properties very similar to those of the green plant enzymes, but functions with NAD (NAD^+ and NADH) as well as with NADP ($NADP^+$ and NADPH). However, it has been found that $NADP^+$ (or NADPH) is the better electron acceptor (or donor) for the enzyme when the concentration of pyridine nucleotide used is lower than about 1.0 mM, while NAD^+ (or NADH) is the better electron acceptor (or donor) when pyridine nucleotide is used at concentrations higher than about 1.0 mM.

MATERIALS AND METHODS

Materials

FAD, FMN, NADH, NAD^+, NADPH, $NADP^+$, 2′-AMP, 3′-AMP, 5′-AMP, sodium pyruvate, glucose 6-phosphate, D-alanine, D-amino acid oxidase, catalase, lactate dehydrogenase, *p*-chloromercuribenzoic acid, quinacrine hydrochloride (atebrin hydrochloride) and rotenone were purchased from Sigma Chemical Company (U.S.A.), yeast glucose-6-phosphate dehydrogenase from Boehringer Mannheim Corporation (Germany), DEAE-cellulose from Serva Entwicklungslabor (Germany), Sephadex G-100 from Pharmacia (Sweden), and benzyl viologen from British Drug Houses, Ltd (England).

Spinach chloroplasts and spinach NADP reductase, and spinach ferredoxin were prepared by the methods of Shin *et al.* [2]and Tagawa and Arnon[8], respectively. Cytochrome *c*-555, and cytochromes *c*-553 and *c*-551 from *C. thiosulfatophilum* were prepared according to the methods established in our laboratory[9], and by the method of Meyer *et al.*[10], respectively. Cytochrome *c*-550 (*Saccharomyces oviformis*)[11] and cytochrome *c*-551 (*Rhodospirillum rubrum*) (cytochrome c_2)[12] were supplied by Sankyo Co., Ltd (Tokyo, Japan), and by Dr T. Horio (Institute for Protein Research, Osaka University, Japan), respectively.

Chlorobium thiosulfatophilum

The strain of *C. thiosulfatophilum* (NCIB 8346) used in the present studies was kindly supplied by Drs J. H. Mathewson (San Diego State College, Calif., U.S.A.) and T. Meyer (University of California, San Diego, U.S.A.). The organism was grown in Larsen's medium[13] for 5 days as described previously[9]. The harvested cells were stored at −20 °C until use.

Assays of enzymatic activities

NAD(P)H:cytochrome *c* reductase activity was determined according to Marrè and Servettaz[14], and NAD(P)H:DCIP reductase and pyridine nucleotide transhydrogenase activities by the methods as described by Yamanaka and Kamen[4]. NAD(P)H:benzyl viologen reductase activity was determined anaerobically in an Ar atmosphere. For this purpose, Thunberg-type cuvettes were used, and the increase in the absorbance at 550 nm was followed spectrophotometrically. The

standard reaction mixture contained 100 μmoles Tris–HCl buffer, pH 8.7, 0.1 μmole NADPH or NADH, 0.5 μmole benzyl viologen and an appropriate amount of the enzyme, in a total volume of 2.0 ml. NAD(P) reductase activity was determined with $Na_2S_2O_4$-reduced benzyl viologen[4] or with illuminated chloroplasts[2] as the electron donor.

Determination of flavin

To an NAD(P) reductase solution, an equal volume of 1 M trichloroacetic acid was added and the resulting turbid mixture was centrifuged at $3000 \times g$ for 2 min to separate the precipitate. The supernatant thus obtained, after removal of trichloroacetic acid by extraction with ethyl ether, was examined by the method of DeLuca *et al.*[15] to determine if it activated the apo-enzyme of D-amino acid oxidase which has been known to be activated only by FAD. Further, the precipitate which was obtained by the above trichloroacetic acid-treatment was suspended in 0.1 M Tris–HCl buffer and the pH of the resulting suspension was adjusted to 7 with 2 M NaOH. With the protein suspension thus obtained, an examination was made of which flavin (FAD or FMN) could activate the benzyl viologen reductase activity of the protein moiety derived from the NAD(P) reductase.

Physical and chemical measurements

Determinations of spectral properties were performed at 20 °C in a Cary recording spectrophotometer, model 14 or 15, using cuvettes with a 1-cm light path. The millimolar extinction coefficients used here were; 29.0 for the reduced form of *S. oviformis* cytochrome *c* (at α-peak)[16,*], 20.0 for DCIP (at 600 nm)[17], 6.22 for NADPH and NADH (at 340 nm)[18], and 9.0 for reduced form of benzyl viologen (at 550 nm)[4]. Protein contents were determined according to Lowry *et al.*[19]. Chlorophyll contents were determined by the method of Arnon[20].

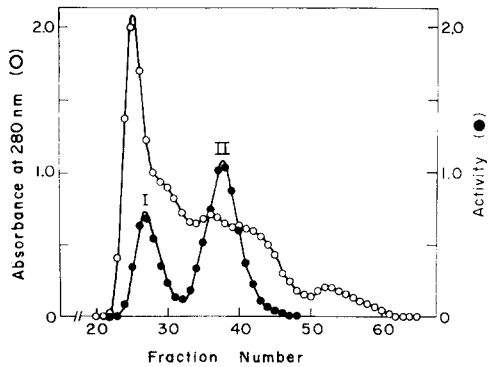
RESULTS

Purification of NAD(P) reductase

The cell pastes (about 150 g wet wt) were treated with 3 l acetone (at approximately 0 °C for 5 min), separated by filtration, and dried in air at room temperature. The weight of the acetone-dried cells was about 30 g. All subsequent operations were conducted in a cold room at 2–4 °C. The acetone-dried cells were suspended in 500 ml of 10 mM Tris–HCl buffer, pH 8.5, which was 10% saturated with $(NH_4)_2SO_4$. The resulting suspension was centrifuged at $12000 \times g$ for 20 min after standing overnight with continuous stirring. The supernatant thus obtained was fractionated with $(NH_4)_2SO_4$; the precipitate formed between 40 and 80% saturation was collected by centrifugation and dissolved in 0.1 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl. The solution thus obtained was loaded onto a DEAE-cellulose column (5 cm × 10 cm) which had been equilibrated with 0.1 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl, after overnight dialysis against the same buffer as used for the chromatography. After the column had been successively

* As *S. oviformis* cytochrome *c* possesses the same primary structure as *S. cerevisiae* cytochrome *c*, the extinction coefficient for the latter cytochrome was used.

washed with 0.1 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl and with 0.1 M Tris–HCl buffer, pH 8.5, containing 0.15 M NaCl, NAD(P) reductase was eluted with 0.1 M Tris–HCl buffer, pH 8.5, containing 0.2 M NaCl. The resulting eluate was fractionated with $(NH_4)_2SO_4$ and the precipitate formed between 40 and 65% saturation was collected by centrifugation. The precipitate thus obtained was dissolved in 0.1 M Tris–HCl buffer, pH 8.5, and the resulting solution was subjected to further purification by chromatography with Sephadex G-100. The elution pattern with the Sephadex G-100 column showed two peaks with NADH: DCIP reductase activity (Fig. 1). When Peak I was rechromatographed on the Sephadex G-100 column, it was split into the two peaks already seen in the first chromatography, judging from the NADH:DCIP reductase activity. From the above gel filtration on the Sephadex G-100 column, the molecular weights of the materials contained in Peaks I and II were determined as being about 90000 and 45000, respectively, according to the method of Andrews[21]. It seemed that



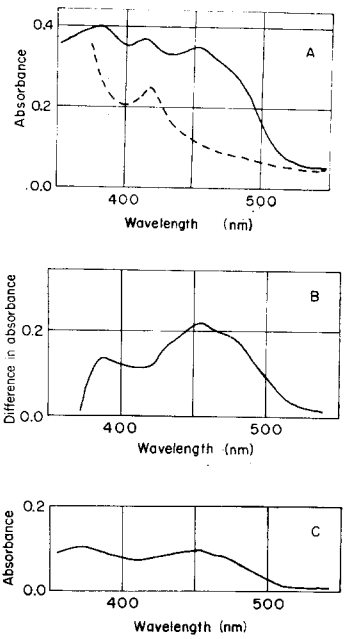


Fig. 1. Elution pattern of NAD(P) reductase during chromatography on a Sephadex G-100 column. 30 mg of the protein dissolved in 0.1 M Tris–HCl buffer, pH 8.5, were loaded onto the Sephadex G-100 column (2.0 cm × 70 cm) which had been equilibrated with 0.1 M Tris–HCl buffer, pH 8.5. The eluate was collected as 2.0-ml fractions. The activities of the enzyme fractions were determined by NADH–DCIP reduction and expressed as $-\Delta A_{600\,nm}$ per min. The reaction mixture for the determination of this activity contained 50 μmoles of Tris–HCl, pH 7.5, 0.05 μmole of DCIP, 0.20 μmole of NADH and 0.05 ml of each fraction, in a total volume of 1.0 ml.

Fig. 2. Absorption spectra of NAD(P) reductase. (A) The enzyme dissolved in 0.1 M Tris–HCl buffer, pH 8.5, at the concentration of 1.3 mg protein/ml. ——, oxidized; - - - - - -, reduced with $Na_2S_2O_4$. (B) Difference spectrum of the enzyme, oxidized *minus* reduced. (C) Trichloroacetic acid extract of the enzyme. As an equal volume of trichloroacetic acid was added to the enzyme solution, the concentration of the substance which was extracted was approximately equivalent to half of the initial enzyme concentration.

Peaks I and II contained the same enzyme but were of different molecular species, as the eluates belonging to the two peaks showed qualitatively the same enzymatic activity in each reaction such as NAD(P)H–cytochrome *c* reduction, NAD(P)H–DCIP reduction, NAD(P)H–benzyl viologen reduction, reduced benzyl viologen–$NAD(P)^+$ reduction and transhydrogenation. Peak II showed greater total activity in each enzymatic reaction mentioned above than did Peak I, and the specific activity was also higher with the Peak II than with the Peak I. Therefore, in the present studies we used the eluate containing Peak II for the determination of the various properties. It was also confirmed with Peak II that the peak of enzymatic activity coincided with that of the absorbance at 280 nm after rechromatography on the Sephadex G-100 column, although the elution pattern is not shown in the figure.

Spectral properties of NAD(P) reductase

The NAD(P) reductase preparation was yellow in colour and showed an absorption spectrum similar to that of a flavoprotein (Fig. 2A) although it was contaminated with a small amount of a cytochrome as seen from the $Na_2S_2O_4$-reduced preparation; there were peaks at 455 nm, 385 nm and 412 nm, and a shoulder around 485 nm. When $Na_2S_2O_4$ was added to the enzyme solution, the peak at 455 nm disappeared, while a small peak appeared at 415 nm. The latter peak seemed to be attributable to the Soret peak of a cytochrome contaminating the preparation. The reduced enzyme was autoxidizable; thus the original absorption spectrum readily appeared on reoxidation by aeration, but the contaminating cytochrome remained reduced. The difference spectrum (reoxidized *minus* reduced) showed the absorption spectrum of a flavoprotein (Fig. 2B).

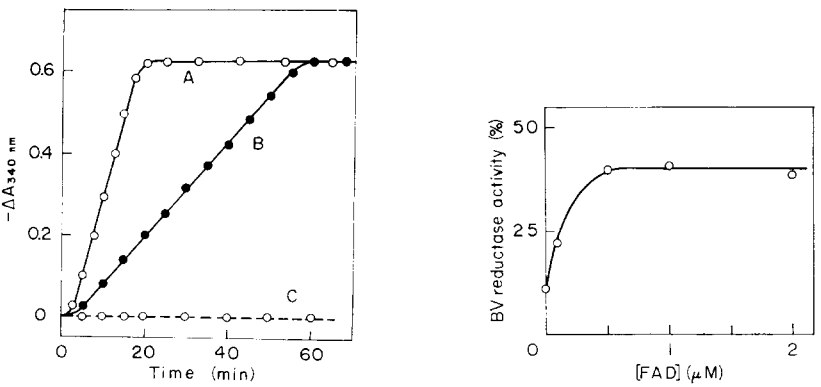


Fig. 3. Activation of the apo-enzyme of D-amino acid oxidase by flavins. A, authentic FAD (final concentration, 0.5 μM); B, trichloroacetic acid extract of the NAD(P) reductase (trichloroacetic acid had been removed by ethyl ether extraction). $A_{450\,nm}$ of the extract was about 0.005 in the reaction mixture; this is equivalent to a final concentration of 0.45 μM assuming that the yellow substance in the extract was FAD; C, authentic FMN (final concentration 0.5 μM).

Fig. 4. Effect of FAD concentration on activation of the trichloroacetic acid-treated NAD(P) reductase. The trichloroacetic acid-treated enzyme, prepared as described in the text, was incubated for 15 min at 20 °C in the presence of FAD at the concentrations indicated. Then the benzyl viologen reductase activity of the trichloroacetic acid treated-enzyme was determined and expressed as a percentage of the activity of the non-treated enzyme.

The prosthetic group of NAD(P) reductase

When an equal volume of 1 M trichloroacetic acid was added to the NAD(P) reductase solution and the resulting mixture was centrifuged, the colourless protein moiety was precipitated while the supernatant became pale yellow. The supernatant, after removal of the trichloroacetic acid by extraction with ethyl ether, showed the absorption spectrum of a typical flavin (Fig. 2C) and activated the apo-enzyme of D-amino acid oxidase (Fig. 3). The activity with the neutralized trichloroacetic acid-extract was a little lower than that with FAD although the flavins were added at approximately the same concentration. This is probably attributable to a partial decomposition by the trichloroacetic acid treatment of the flavin derived from the enzyme. The protein moiety of the NAD(P) reductase obtained by trichloroacetic acid treatment was suspended in 0.1 M Tris–HCl buffer at a concentration of about 0.75 μM on the basis of the original enzymatic activity, and the pH of the resulting suspension was adjusted to 7 with 2 M NaOH. The suspension thus obtained partially recovered enzymatic activity on addition of FAD (final concentration, 1.0 μM) (Fig. 4 and Table I), but was not affected by the addition of FMN. As judged from the above facts, the prosthetic group of the NAD(P) reductase may be assumed to be FAD.

TABLE I

ACTIVATION OF TRICHLOROACETIC ACID-TREATED NAD(P) REDUCTASE BY FLAVIN

The activation was monitored by NADPH–benzyl viologen reduction. The reaction mixture contained, besides added FAD or FMN, 100 μmoles of Tris–HCl, pH 8.7, 0.1 μmole of NADPH, 0.5 μmole of benzyl viologen, 100 μg of the enzyme or the trichloroacetic acid-treated enzyme in a total volume of 2.0 ml. The reactions were performed anaerobically under Ar atmosphere using Thunberg-type cuvettes at 20 °C.

Compounds added	$\triangle A_{550\,nm}$ *per min per mg protein*	*Activity* (%)*
With the native enzyme		
None	2.30	100
FMN (1.0 μM)	2.28	99.2
FAD (1.0 μM)	2.25	97.8
With the trichloroacetic acid-treated enzyme		
None	0.25	10.9
FMN (1.0 μM)	0.25	10.9
FAD (1.0 μM)	0.94	40.8

* The activity of the native enzyme without addition of FAD or FMN was taken as 100%.

Light-dependent NAD(P)$^+$ reduction by NAD(P) reductase with photoreduced spinach ferredoxin

Fig. 5 shows the time course of light-dependent NAD(P)$^+$ reduction by the NAD(P) reductase with illuminated spinach chloroplasts and added spinach ferredoxin. The reactions were performed under anaerobic conditions using Thunberg-

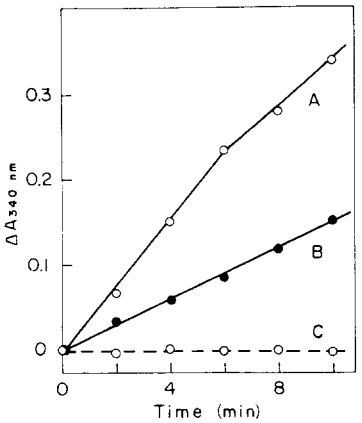


Fig. 5. Time course of light-dependent $NAD(P)^+$ reduction by NAD(P) reductase and photoreduced spinach ferredoxin. The complete reaction mixture contained, in a total volume of 2.5 ml, 150 μmoles of Tris–HCl, pH 8.0, the suspension of chloroplasts containing 30 μg of chlorophyll, 100 μg of spinach ferredoxin, 1.0 μmole of $NADP^+$ or NAD^+, 0.2 μmole of DCIP, 10 μmoles of ascorbate and 707 μg of the enzyme. The reaction was anaerobically performed at 20 °C in a Thunberg-type cuvette under illumination (10000 lux). A, light, $+NADP^+$; B, light, $+NAD^+$; C, dark, $+NADP^+$ or $+NAD^+$.

type cuvettes. $NADP^+$ was reduced about twice as rapidly as NAD^+ at the concentrations used in the experiments (see below). As Table II shows, the photoreduction of pyridine nucleotides required added spinach ferredoxin and an electron donor (ascorbate *plus* DCIP) as well as the NAD(P) reductase. The reduction was also dependent on chloroplasts which catalyzed the photoreduction of ferredoxin. It was confirmed that in the $NADP^+$ reduction the increased absorbance at 340 nm decreased to around the original level quite rapidly when air was introduced and

TABLE II

REDUCTION OF $NAD(P)^+$ WITH ILLUMINATED CHLOROPLASTS

The composition of the complete reaction mixture was the same as for Fig. 5.

Compounds omitted	$\Delta A_{340\,nm}$ *per 10 min*
In the light	
None	0.40
Ferredoxin	0.06
$NADP^+$	0.00
Ascorbate and DCIP	0.15
Enzyme	0.09
Chloroplasts	0.00
$NADP^+$, *plus* NAD^+	0.14
In the dark	
None	0.00

a small amount of spinach NADP reductase was added to the reaction mixture at the end of the reaction. Also in the NAD^+ reduction, the immediate decrease of the increased absorbance at 340 nm was observed on addition of rabbit lactate dehydrogenase and pyruvate.

When ferredoxin isolated from *C. thiosulfatophilum*[22] was used in place of spinach ferredoxin in the above experiments, the rate of the photoreduction of $NAD(P)^+$ was slower than that with spinach ferredoxin. This is probably attributable to the lability of *C. thiosulfatophilum* ferredoxin, *i.e.* the major part of the ferredoxin preparation of this organism which we used might have been denatured.

Reduction of $NAD(P)^+$ by NAD(P) reductase with reduced benzyl viologen as the electron donor

The NAD(P) reductase preparation reduced $NAD(P)^+$ with reduced benzyl viologen as the electron donor under anaerobic conditions. The figures shown in Table III are the averages of three experiments. In the reactions, $NADP^+$ was a more efficient electron acceptor than NAD^+. The quantity of the reduced pyridine nucleotide formed was in good agreement with that expected theoretically from the amount of reduced viologen consumed; as benzyl viologen is a one-electron carrier, the 2 moles of it are expected to reduce 1 mole of pyridine nucleotide. The pH optimum in the $NAD(P)^+$ reduction with the reduced benzyl viologen was at pH 8.7 and the K_m values for $NADP^+$ and NAD^+ were 59 μM and 200 μM, respectively.

TABLE III

PYRIDINE NUCLEOTIDE REDUCTION BY NAD(P) REDUCTASE WITH REDUCED BENZYL VIOLOGEN

The complete reaction mixture contained 150 μmoles of Tris–HCl, pH 8.7, 1.0 μmole of benzyl viologen, 0.5 μmole of $NADP^+$, and 707 μg of the enzyme, in a total volume of 2.5 ml. Benzyl viologen was carefully reduced by a small amount of $Na_2S_2O_4$ before the reactions were started. The reactions were anaerobically carried out at 20 °C.

Compounds omitted	$-\Delta A_{550\,nm}$ *per min*	*Reduced benzyl viologen consumed per min (nmoles)*	$\Delta A_{340\,nm}$ *per min*	*Reduced pyridine nucleotide formed per min (nmoles)*
None	0.416	116	0.125	50
Enzyme	0.035	9.7	0.006	2.4
Pyridine nucleotide	0.030	8.3	0.005	2.0
$NADP^-$, *plus* NAD^-	0.266	74	0.086	34

NAD(P)H–cytochrome c reduction by NAD(P) reductase

The NAD(P) reductase preparation reduced cytochrome *c*-550 (*S. oviformis*) (a mammalian-type cytochrome *c*) with NAD(P)H as the electron donor. Also in this reaction, NADPH was more efficient than NADH. When cytochrome *c*-555 (*C. thiosulfatophilum*) or cytochrome *c*-551 (*Rhodospirillum rubrum*) were

TABLE IV

REDUCTION CATALYSED BY NAD(P) REDUCTASE OF CYTOCHROME *c* WITH NAD(P)H

The standard reaction mixture contained 50 μmoles of Tris–HCl, pH 8.7, 0.05 μmole of NADPH or NADH, 0.05 μmole of yeast cytochrome *c* and 8.85 μg of the enzyme, in a total volume of 1.0 ml. The reactions were performed at 20 °C.

Compounds added	*Concentration* (μM)	$\triangle A_{550\,nm}$ *per min*	
		NADPH	*NADH*
None	—	0.430	0.134
$NADP^+$	57.7	0.245	0.086
$NADP^+$	263	0.066	0.033
$NADP^+$	577	0.050	0.020
NAD^+	577	0.220	0.065

TABLE V

REDUCTION CATALYSED BY NAD(P) REDUCTASE OF CYTOCHROMES *c* DERIVED FROM VARIOUS ORGANISM

The reaction conditions were the same as for Table IV except that various kinds of cytochromes *c* were used in place of yeast cytochrome *c*.

Source of cytochrome c	*Concentration* (μM)	*α-Peak* (*nm*)	$\triangle A_\alpha$ *per min*
Saccharomyces oviformis	50	550	0.430
Rhodospirillum rubrum	50	551	0.502
Chlorobium thiosulfatophilum	50	555	0.340
Chlorobium thiosulfatophilum	50	551	0.025
Chlorobium thiosulfatophilum	50	553	0.010

TABLE VI

EFFECT OF VARIOUS INHIBITORS ON NADPH–CYTOCHROME *c* REDUCTION CATALYZED BY NAD(P) REDUCTASE

The standard reaction conditions were the same as for Table IV.

Inhibitors	*Concentration* (*mM*)	*Inhibition* (%)
2′-Adenylic acid	2.0	44
3′-Adenylic acid	2.0	32
5′-Adenylic acid	2.0	25
$NADP^+$	0.05	45
$NADP^+$	0.5	86
NAD^+	0.5	48
p-Chloromercuribenzoate	0.05	50
$ZnSO_4$	0.1	44
Rotenone	0.05	52
Atebrin hydrochloride	0.05	70

used as the electron acceptor, the reactions proceeded at the rates similar to the case of yeast cytochrome *c*, whereas cytochromes *c*-551 and *c*-553 of *C. thiosulfatophilum* were hardly reduced by the enzyme with NADPH of NADH (Table V). These NAD(P)H:cytochrome *c* reductase activities were strongly inhibited by $NADP^+$, while NAD^+ had little effect on them (Table IV). The effects of various inhibitors on the enzymatic reduction of cytochrome *c* were tested (Table VI). 2′-Adenylic acid inhibited the reaction more strongly than 3′-adenylic acid and 5′-adenylic acid. Other effective inhibitors tested were *p*-chloromercuribenzoate, $ZnSO_4$, rotenone and atebrin hydrochloride. The pH optimum in the NAD(P)H–cytochrome *c* reduction with yeast cytochrome *c* was at pH 8.7 and the K_m values for NADPH and NADH were 13 μM and 200 μM, respectively. These values did not vary with the concentration of the cytochrome *c* used as the electron acceptor.

Other enzymatic activities of NAD(P) reductase

The NAD(P) reductase preparation reduced DCIP and benzyl viologen with NAD(P)H. In these reactions, NADH functioned less efficiently than NADPH as the electron donor. The enzyme preparation showed also a pyridine nucleotide transhydrogenase activity, *i.e.* NAD^+ was reduced in the presence of the enzyme with glucose-6-phosphate dehydrogenase and glucose 6-phosphate as a continuous NADPH-forming system. However, as far as tested, the reverse reaction was not catalyzed by the enzyme at all, using lactate dehydrogenase and lactate as a continuous NADH-forming system. In Table VII, we show the reaction rates and K_m values for the various reactions catalyzed by the NAD(P) reductase preparation.

TABLE VII

COMPARISON OF VARIOUS ENZYMATIC ACTIVITIES OF NAD(P) REDUCTASE

Reactions	*pH*	*Reaction rate (moles/min per mole of flavin of the enzyme)*		K_m** *(mM)*
		*At experimental conditions**	*V*	
NADPH→cytochrome *c*	8.7	277 (0.05 mM)	321	0.013 (NADPH)
NADH→cytochrome *c*	8.7	134 (0.05 mM)	461	0.167 (NADH)
NADPH→DCIP	7.5	259 (0.05 mM)	395	0.031 (NADPH)
NADH→DCIP	7.5	91.2 (0.05 mM)	528	0.670 (NADH)
NADPH→NAD^+	7.5	110 (0.1 mM)	123	0.031 (NADPH)
		(1.0 mM)		0.140 (NAD^+)
NADPH→benzyl viologen	8.7	765 (0.05 mM)	2380	0.109 (NADPH)
NADH→benzyl viologen	8.7	119 (0.05 mM)	4020	1.43 (NADH)
Reduced benzyl viologen→$NADP^+$	8.7	12.6 (0.10 mM)	272	0.059 ($NADP^+$)
Reduced benzyl viologen→NAD^+	8.7	5.35 (0.10 mM)	320	0.250 (NAD^+)
Photoreduction of $NADP^+$	8.0	6.10 (0.20 mM)	N.D.***	N.D.***
Photoreduction of NAD^+	8.0	2.26 (0.20 mM)	N.D.***	N.D.***

* The concentration of pyridine nucleotide used in each reaction is indicated in the parentheses.

** K_m values are for the compounds which are indicated in the parentheses.

*** Not determined.

The DCIP reductase activity was inhibited competitively by $NADP^+$ and 2'-adenylic acid; K_i values for $NADP^+$ and 2'-AMP were 37 μM and 0.9 mM, respectively (Fig. 6). The transhydrogenase activity was also inhibited competitively by 2'-adenylic acid (K_i, 0.2 mM) (Fig. 7).

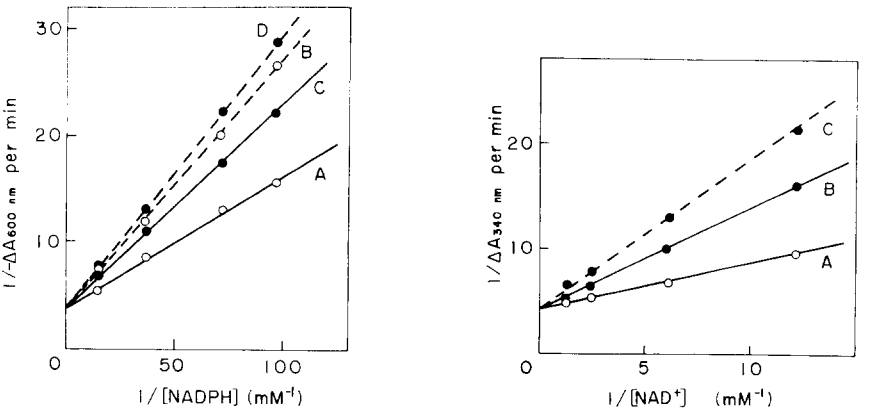


Fig. 6. Competitive inhibition of NADPH:DCIP reductase activity by $NADP^+$ and 2'-AMP as demonstrated by Lineweaver–Burk plots. The standard assay mixture contained 50 μmoles of Tris–HCl, pH 7.5, 0.05 μmole of DCIP, 8.85 μg of the NAD(P) reductase and various concentrations of NADPH, in a total volume of 1.0 ml. A, control; B, 33.3 μM $NADP^+$; C, 0.5 mM 2'-AMP; D, 1.0 mM 2'-AMP.

Fig. 7. Competitive inhibition of pyridine nucleotide transhydrogenase activity by 2'-AMP as demonstrated by Lineweaver–Burk plots. The standard assay mixture contained 50 μmoles of Tris–HCl, pH 7.5, 50 μmoles of glucose 6-phosphate, 20 μg of glucose -6-phosphate dehydrogenase, 0.1 μmole of $NADP^+$, 83.3 μg of the NAD(P) reductase and various concentrations of NAD^+ in a total volume of 1.0 ml. A, control; B, 0.25 mM 2'-AMP; C, 0.5 mM 2'-AMP.

Effect of concentration of pyridine nucleotide on NAD(P) reductase

As Fig. 8 shows, the concentration of pyridine nucleotide greatly affected the NAD(P)H:DCIP reductase activity of the enzyme; under conditions where the concentration of pyridine nucleotide was lower than approx. 1 mM, the enzyme utilized NADPH more rapidly than NADH, while in the presence of the pyridine nucleotide of concentrations higher than approx. 1 mM, the enzyme reduced the

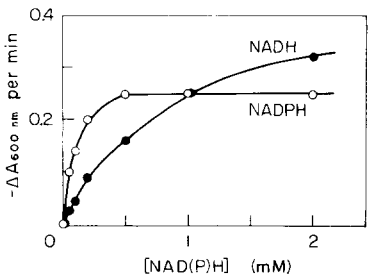


Fig. 8. Influence of the pyridine nucleotide concentration on the NAD(P)H:DCIP reductase activity of NAD(P) reductase. The reaction mixture contained 50 μmoles of Tris–HCl, pH 7.5, 0.05 μmole of DCIP, 5.45 μg of the NAD(P) reductase and various concentrations of NADPH or NADH, in a total volume of 1.0 ml.

dye more rapidly with NADH than with NADPH. Such concentration dependency was observed also in NAD(P)H:cytochrome *c* reductase, NAD(P)H:benzyl viologen reductase and reduced benzyl viologen:$NAD(P)^+$ reductase reactions. Thus, when the V values of each reaction were compared with respect to the types of pyridine nucleotides, they were larger with NAD^+ or NADH than with $NADP^+$ or NADPH (Table VII).

DISCUSSION

The mechanisms of the photosynthetic reduction of pyridine nucleotide in green plants, *i.e.* in higher plants and algae, have been well established; ferredoxin and NADP reductase are essential for the reduction, and $NADP^+$ is exclusively reduced[1–4]. In the photosynthetic bacteria, the mechanisms of the photosynthetic reduction of pyridine nucleotide have not been sufficiently elucidated. An NAD(P) reductase has been isolated from *R. palustris* and its properties studied in detail[5]. This enzyme utilizes $NADP^+$ or NADPH more efficiently than NAD^+ or NADH. This means that the specificity of the bacterial enzyme for pyridine nucleotide is not so strict as that of the NADP reductases derived from higher plants and algae. Our interest has been to know whether the specificity for pyridine nucleotide of the pyridine nucleotide reductase is related to the evolutionary degree of the parent organisms; it has been expected that the enzyme derived from the most primitive photosynthetic organism may utilize only NAD^+ or NADH.

In the present studies, we have obtained a highly purified preparation of an NAD(P) reductase from *C. thiosulfatophilum* and determined its properties. The enzyme is very similar to the spinach enzyme in various properties; it catalyzes the reduction of $NAD(P)^+$ with illuminated chloroplasts and ferredoxin or with reduced benzyl viologen. It also catalyzes NAD(P)H–cytochrome *c* reduction, NAD(P)H–DCIP reduction, NAD(P)H–benzyl viologen reduction and a pyridine nucleotide transhydrogenation. In these reactions the enyzme utilizes $NADP^+$ or NADPH more efficiently than NAD^+ or NADH, which went against our earlier expectation. However, a very interesting phenomenon has been discovered in the present investigation; under conditions where the concentration of pyridine nucleotide is lower than approx. 1 mM, the NAD(P) reductase utilizes $NADP^+$ or NADPH more efficiently than NAD^+ or NADH, whereas in the presence of nucleotide at concentrations higher than approx. 1 mM the enzyme reacts more rapidly with NAD^+ or NADH than with $NADP^+$ or NADPH. Thus, the K_m values of the enzyme for NAD^+ or NADH are about ten times greater than those for $NADP^+$ or NADPH. Similar results have been also obtained with an NAD(P) reductase derived from *R. palustris*[5]. In general, the concentrations of $NAD(P)^+$ and NAD(P)H used by many investigators for their experiments are around 3 mM, as seen in literature. For example, Buchanan and Evans[23] have claimed that NAD^+ is photosynthetically reduced more rapidly than $NADP^+$ with the illuminated cell-free extracts of *C. thiosulfatophilum*. However, they have used the pyridine nucleotides at the concentration of 3.3 mM. In our preliminary experiments, the concentrations of NADP ($NADP^+$ *plus* NADPH) and NAD (NAD^+ *plus* NADH) in the cells of the bacterium were 14 μM and 16 μM, respectively, being in good agreement with those found with green plants[24]. Therefore, it seems likely that

the NAD(P) reductase of *C. thiosulfatophilum* photosynthetically reduces $NADP^+$ more rapidly than NAD^+ *in vivo*. In any case, NADPH will be primarily formed in the organism during the photosynthetic reduction of pyridine nucleotide. The fact that the kind of pyridine nucleotide which functions more efficiently with the enzyme varies with the concentration of the nucleotide used may play an important role in the regulation of biosynthetic systems in the bacterium.

Three types of *c*-type cytochromes, cytochromes *c*-551, *c*-553 and *c*-555 (refs 9 and 10) have been isolated from *C. thiosulfatophilum*. Cytochrome *c*-555 among them seems to be isologous to the *f*-type cytochrome of algae as judged from its spectral and enzymatic properties[7,9]. This idea appears to be supported also by the fact that only cytochrome *c*-555 among these three types of *c*-type cytochromes reacts with the NAD(P) reductase of the bacterium, as the *f*-type cytochrome of *Navicula pelliculosa* is easily reducible by the algal NADP reductase in the presence of NADPH without the addition of ferredoxin[4].

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