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LIGHT-INDUCED CONVERSION OF NAD^+ TO $NADP^+$ IN *CHLORELLA* CELLS

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Illumination of *Chlorella vulgaris* 11 h cells which had been preincubated in the dark caused conversion of NAD^+ to $NADP^+$. This conversion was saturated at relatively low light intensity. Both red and blue light were effective but green and far-red light showed only slight effect. 3-(4-Chlorophenyl)-1,1-dimethyl urea, disalicylidene propanediamine and carbonylcyanide 3-chlorophenylhydrazone inhibited the light-induced conversion. No conversion was induced by illumination with far-red light in an N_2 atmosphere, although the ATP-level rose quickly. Likewise, chlorophyll-less yellow mutant strains, *C. vulgaris* 211-11 h/20 cells did not show light-induced conversion. These results indicate that the conversion of NAD^+ to $NADP^+$ is associated with both photosynthetic electron transport and photophosphorylation. Active conversion was observed during the induction period of CO_2 fixation.

Introduction

Light-induced conversion of NAD^+ to $NADP^+$ was observed in *Chlorella ellipsoidea* cells [1,2] and higher plant leaves [3,4]. Oh-hama et al. [5] isolated NAD kinase from sonically disintegrated cells of *Chlorella*. Since they showed photosynthetic phosphorylation activity in the presence of phenazine methosulfate or flavine mononucleotide, they assumed that NAD kinase in *Chlorella* catalyzes the phosphorylation of NAD^+ to $NADP^+$ using photochemically produced ATP in the light.

Since the light-induced conversion of NAD^+ to $NADP^+$ was discovered, more than 20 years ago, the mechanism of this reaction had been left without further investigation. The present study was designed to elucidate the relationship between this conversion process and photosynthesis. The results indicated that the conversion reaction is dependent on both photosynthetic electron transport and photophosphorylation.

Materials and Methods

Chlorella cells

Chlorella vulgaris 11 h cells were cultured as described [6] until the cell density reached 10 ml packed cell volume/liter. The cells were then harvested by centrifugation 2 h after the start of the last light period. After washing with deionized water, the cells were suspended in 50 mM Hepes-KOH buffer (pH 7.6). Unless otherwise mentioned, the cell density was 15 ml packed cell volume/l.

Chlorophyll-less mutant, *C. vulgaris* 211-11 h/20 cells were cultured for 7 days at 30°C in the dark and in the presence of glucose as described [7]. Harvested

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Abbreviations: Bicine, *N,N*-bis(2-hydroxy)glycine; CCCP, carbonylcyanide 3-chlorophenylhydrazone; CMU, 3-(4-chlorophenyl)-1,1-dimethylurea; DSPD, disalicylidene propanediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide.

cells were suspended under the same conditions as used for the wild type cells.

Extraction and assay of nicotinamide coenzymes

A portion of *Chlorella* cell suspension was quickly taken out to a test tube containing either HCl or NaOH (final concentration, 0.1 M) which had been kept in a boiling water bath. The resulting mixtures were quickly heated for 2 min in a boiling water bath. After immediate cooling, the suspensions were centrifuged and the supernatants were used for the assay of nicotinamide coenzymes. The oxidized forms of the coenzymes were extracted by the acid treatment, while the alkaline extract contained only the reduced forms. The extracts could be stored in an ice-bath for up to 48 h without appreciable breakdown of the coenzymes.

Nicotinamide coenzymes were assayed by an enzymatic cylcin method [8]. To correct any decomposition of coenzyme caused by acid or alkali treatment as well as possible effects of unknown compounds interfering with the enzymatic assay, the authentic coenzyme solution (a), the *Chlorella* cell suspension (b) and the cell suspension containing (a) (termed (c)) were treated with either acid or alkali as described above. Factors calculated from [rate in (c) – rate in (b)]/[rate in (a)] were used for the correction.

Extraction and assay of ATP

For the determination of ATP levels, 0.2 ml of the algal suspension was quickly transferred to a test tube containing 1 ml methanol which had been kept at 60°C. The methanol suspension was shaken well and kept on ice until use. Methanol was removed by the stream of N_2 gas at room temperature, then 1 ml 20 mM Tris-HCl buffer (pH 7.75) containing 2 mM EDTA was added. The suspension was kept in the boiling water-bath to extract ATP. After 2 min, the suspension was centrifuged at 4°C. ATP was determined fluorometrically with 50 µl of the supernatant thus obtained, luciferin-luciferase solution (FLE 50, Sigma Chemical Co.) and CHEM.-GLOW Photometer (American Instrument Co.) [9,10].

Photosynthetic reactions

After preincubation of *Chlorella* cells in the dark, $NaH^{14}CO_3$ was added (final concentration, 8 mM) and light was immediately turned on. The reaction was stopped by the addition of methanol (final concentration, 80%) after 5 min. Aliquots were acidified by acetic acid in vials and kept at 60°C under stream of air for 1 h to remove non-fixed $^{14}CO_2$. The ^{14}C incorporated was determined with a liquid scintillation spectrophotometer (Beckman LS-230). Light-dependent O_2 evolution in the presence of CO_2 or *p*-benzoquinone (Hill reaction) was followed polarographically using a Clark-type oxygen electrode (Rank Brothers, Cambridge).

Light sources

A 500 W xenon lamp was used unless otherwise mentioned. Thermal radiation was eliminated by a flat vessel filled with running water and an infrared reflecting filter ('Cold Filter B', Nihon Shinku Kogaku Co. Ltd., Tokyo). Light intensities were measured with a Spectra Model PR-1000 Photometer/Radiometer (Photo Research, CA).

Chemicals

Nicotinamide coenzymes were products of Oriental Yeast Co., Tokyo. Alcohol and glucose-6-phosphate dehydrogenases, glucose 6-phosphate were purchased from Boehringer Mannheim Yamanouchi Co., Tokyo. MTT, Bicine, Hepes were from Sigma Chemicals Co., St. Louis. ATP and $NaH^{14}CO_3$ were obtained from Kyowa Hakko Kogyo Co., Tokyo and the Radiochemical Centre, Amersham, respectively.

Results

Nicotinamide coenzyme levels in light and darkness

Fig. 1 shows that when *Chlorella vulgaris* 11 h cells which had been preincubated in the dark were illuminated, NAD^+ rapidly decreased and $NADP^+$ rapidly increased. Both coenzymes reached steady-state levels after 10 min of illumination at 81 000 erg/cm^2 per s. NADPH increased and NADH decreased in light, though levels of these coenzymes were small. Upon turning off the light, $NADP^+$ decreased and NAD^+ increased. After a small temporary increase, reduced forms of both coenzymes gradually decreased. The total level of coenzymes was almost constant during the experiment. The time course of decrease in NAD^+-plus-NADH in light was almost a

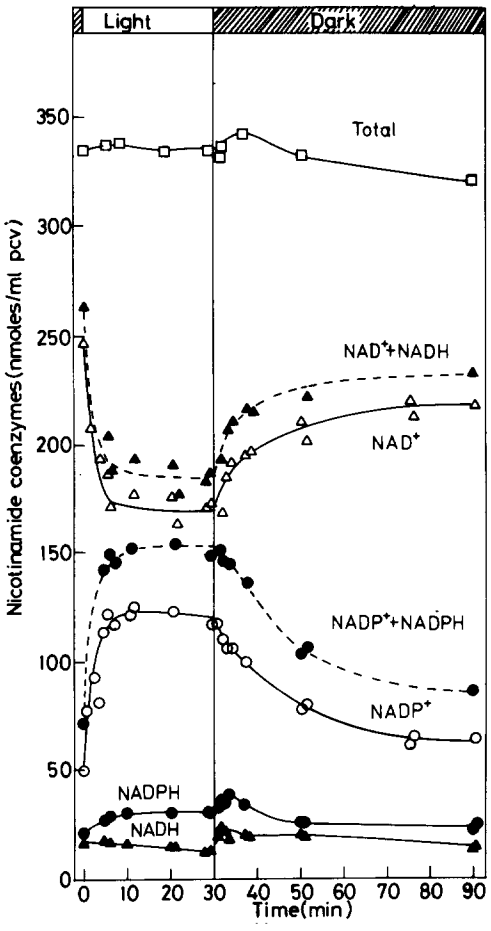

Fig. 1. Nicotinamide coenzyme levels of *Chlorella vulgaris* 11 h cells in light and darkness. 60 ml of cell suspension (5 ml packed cell volume (pcv)/l) in a round and flat glass 'lollipop' vessel (vessel volume, 80 ml) was preincubated for 30 min in the dark at 25°C and then illuminated at 81 000 erg/cm^2 per s. After another 30 min the light was turned off. Bubbling of CO_2-free air was continued throughout the experiment. At scheduled intervals, 1 ml each of the cell suspension was withdrawn with an automatic pipette and injected into HCl or KOH solution to determine the amounts of oxidized or reduced nicotionamide coenzymes.

mirror image of that of increase in NADP$^+$-plus-NADPH. This suggests that NAD$^+$ is converted directly to NADP$^+$ in light. No such quantitative relationship between NAD$^+$-plus-NADH and NADP$^+$-plus-NADPH levels was observed in the dark. Therefore it is unlikely that NADP$^+$ was directly converted to NAD$^+$ in the dark by the reverse reaction to that occurred in light. Changes of nicotinamide coenzyme levels in *C. vulgaris* 11 h in light are consistent with those reported in *C. ellipsoidea* [1,2], except that there was a minor difference in the transient change in NADPH immediately after turning off the light.

Effect of light intensity on the conversion of NAD$^+$ to NADP$^+$

The following experiments were carried out in the presence of CO_2 (ambient air was bubbled through the algal suspension to supply CO_2 unless otherwise mentioned), since it has been reported that the light-induced conversion of NAD$^+$ to NADP$^+$ also occurs in the presence of CO_2 [2].

Both the rate and the magnitude in light-induced conversion of NAD$^+$ to NADP$^+$ were dependent on the light intensity (Fig. 2). Under high light intensity (10 000 erg/cm^2 per s), NAD$^+$ was rapidly converted to NADP$^+$ and attained the steady-state level in less than 10 min, then the NAD$^+$-plus-NADH level was close to the NADP$^+$-plus-NADPH level. On the other hand, the conversion reaction was slower under low light intensity (1000 erg/cm^2 per s) and the steady-state level of NAD$^+$-plus-NADH was much higher than that of NADP$^+$-plus-NADPH.

Fig. 3 shows a light saturation curve for the conversion reaction. In this experiment a thin layer of *Chlorella* cell suspension (thickness, approx. 3 mm) was illuminated to minimize the mutual shading of the algal cells. Time of illumination was 20 min. Steady-state levels of nicotinamide coenzymes were attained in less than 20 min under all light intensities examined. The maximum amount of converted nicotinamide coenzymes (Δ[NADP$^+$ + NADPH]) determined from a double-reciprocal plot was 100 nmol/ml packed cell volume (approx. 10 nmol/mg Chl) and the light intensity to give the half of this value ($L_{0.5}$) was 1400 erg/cm^2 per s.

To compare the dependencies on light intensity for the conversion reaction, Hill reaction and CO_2-dependent O_2 evolution, each $L_{0.5}$ value was determined under the same experimental conditions. In this experiment, the cell suspensions kept in an oxygen-electrode vessel (diameter, 15 mm) was illuminated from the side by a projector (Elmo, Nagoya) equipped with a 300 W lamp and a filter to cut off thermal radiation (see Materials and Methods). The $L_{0.5}$ values for the Hill reaction, photosynthetic O_2 evolution and the conversion reaction of NAD$^+$ to

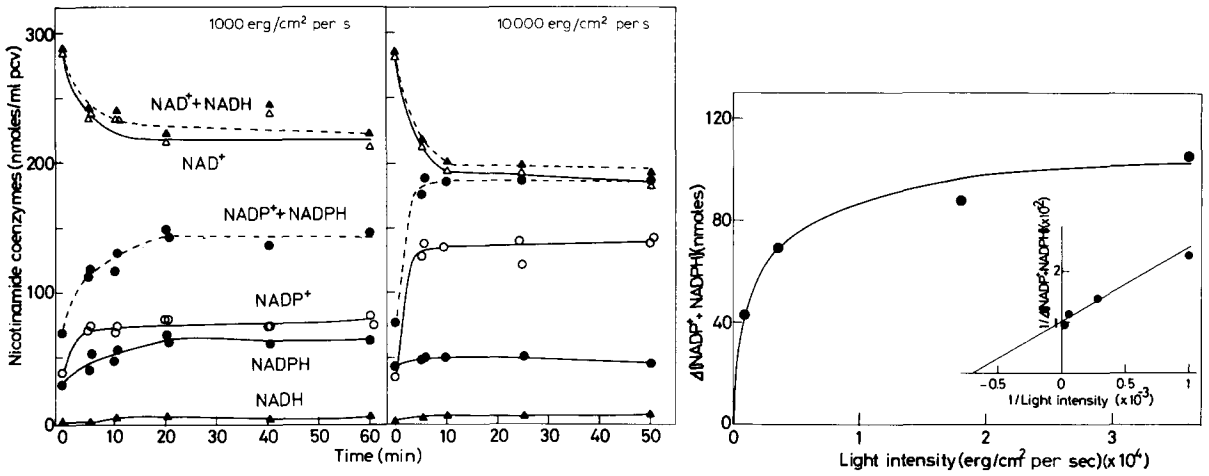

Fig. 2. Light-induced changes of nicotinamide coenzyme levels under high and low light intensities. Experimental conditions were the same as in Fig. 1, except that ambient air was bubbled in place of CO_2-free air and light intensities applied (erg/cm^2 per s) were 1000 (left) and 10 000 (right), respectively.

Fig. 3. Light intensity vs. increase in NADP$^+$-plus-NADPH (Δ[NADP$^+$ + NADPH]. 2 ml *Chlorella* cell suspension (1 ml packed cell volume/l) in a glass cylinder with flat bottom (2.7 cm in diameter) were illuminated from above for 20 min at various light intensities (25°C).

NADP$^+$ were 11 000, 12 000 and 3000 erg/cm^2 per s, respectively.

Effect of the wavelength of incident light on the conversion of NAD$^+$ to NADP$^+$

The effect of the wavelength of incident light on the conversion reaction of NAD$^+$ to NADP$^+$ was examined with monochromatic light (half-band-widths, 10–15 nm) which was obtained by the combined use of an interference filter and an appropriate color filter (Nihon Shinku Kogaku Co. Ltd.). As the absorption spectrum of intact cell suspension showed two main peaks at 440 and 678 nm, and a trough at 550 nm (data not shown), these wavelengths and 712 and 740 nm light, which was maximally absorbed by chlorophyll *a*, were adopted. Red (678 nm) and blue

TABLE I

EFFECTS OF MONOCHROMATIC LIGHT ON THE CONVERSION OF NAD$^+$ TO NADP$^+$ AND ON CO_2 FIXATION

Chlorella cell suspension (1.9 ml; 12.5 ml packed cell volume (pcv)/l) in a glass cylinder with flat bottom (diameter, 2.7 cm) was preincubated in the dark for 2 h at 25°C with continuous shaking. Cells were then illuminated by monochromatic lights from above and the reaction was stopped after 5 min. For CO_2 fixation, 0.1 ml 50 mM $NaH^{14}CO_3$ (spec. act., 2.41 mCi/mmol) was added just prior to illumination. The light intensity was adjusted to give about the same photon number at respective wavelengths. The values in parentheses indicate percentages of those values obtained by illumination at saturating white light ($102 \cdot 10^3$ erg/cm^2 per s).

Wavelength (nm)	Light intensity (erg/cm^2 per s)	$^{14}CO_2$ fixed (μmol/ml pcv per 5 min)	Δ[NADP$^+$ + NADPH] (nmol/nl pcv per 5 min)
440	7 100	7.12 (17.6)	68.2 (80.7)
552	5 600	1.92 (4.7)	16.5 (19.5)
678	4 600	9.82 (24.2)	73.0 (86.4)
712	4 300	0.49 (1.2)	9.0 (10.7)
740	4 200	0.20 (0.5)	5.0 (5.9)

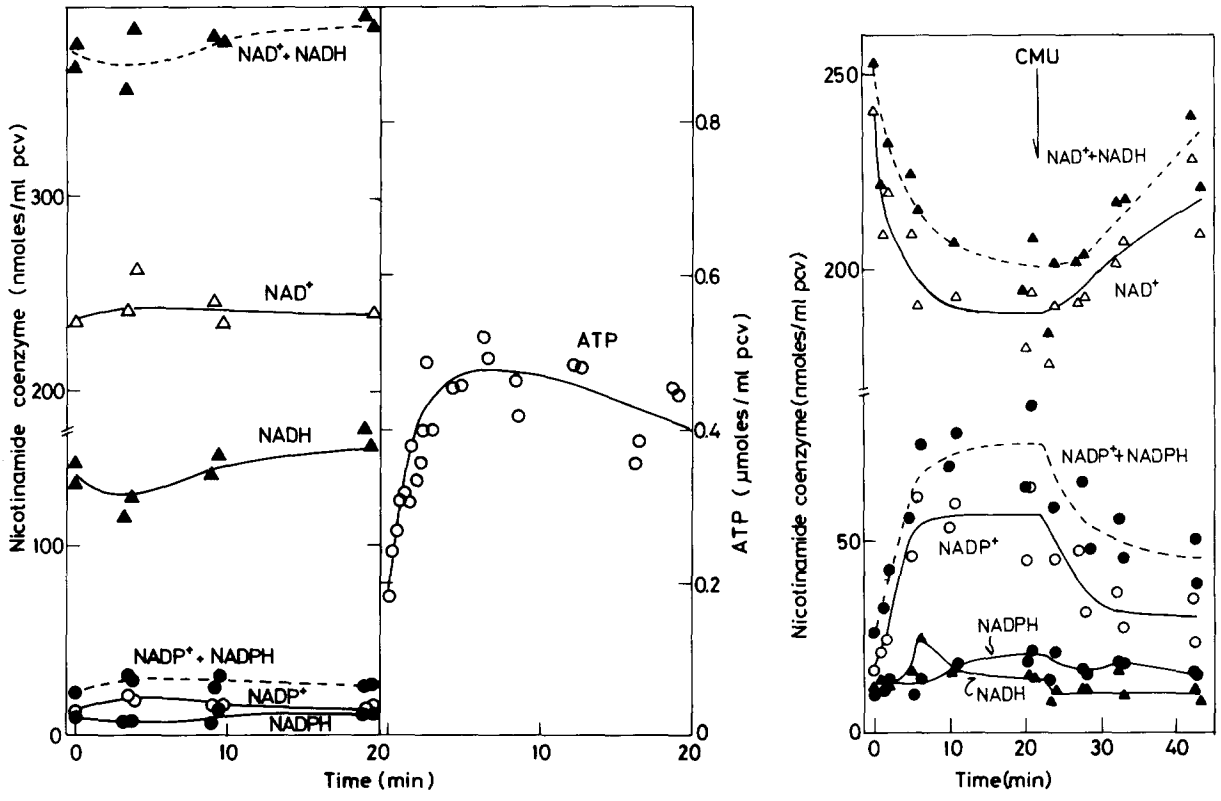

Fig. 4. Effects of far-red light on levels of nicotinamide coenzymes and of ATP. The cell suspension kept in the 'lollipop' at a density of 5 ml packed cell volume (pcv)/l was bubbled with N_2 gas at 550 ml/min in the dark. After 25 min, the 'lollipop' was illuminated with 712 nm light (20 000 erg/cm^2 per s). For other details see Materials and Methods.

Fig. 5. Effect of CMU on the nicotinamide coenzyme levels in light. *Chlorella* cells in the 'lollipop' were preincubated for 30 min in the dark with bubbling of air, and then illuminated at 300 000 erg/cm^2 per s. CMU ($5 \cdot 10^{-5}$ M) was added where indicated by an arrow.

(440 nm) monochromatic light was effective, whereas green (552 nm) and far-red light (712 and 740 nm) was less effective (Table I). Similar responses to the wavelength were observed in the case of CO_2 fixation. To examine the participation of cyclic photophosphorylation in the conversion reaction, far-red light was used under an N_2 atmosphere. Fig. 4 shows that practically no conversion was observed, although the ATP level rose quickly upon illumination at that intensity which saturates for cyclic photophosphorylation ($2 \cdot 10^4$ erg/cm^2 per s) [11]. These results indicate that the cyclic photophosphorylation alone cannot induce the conversion reaction.

Effect of CMU on the light-induced conversion of NAD$^+$ to NADP$^+$

When *Chlorella* cells were illuminated after preincubating for 30 min in the dark in the presence of $5 \cdot 10^{-4}$ M CMU, no change in nicotinamide coenzyme levels was observed (CMU at this concentration inhibited photosynthetic O_2 evolution 90%). Fig. 5 shows that when CMU was added after nicotinamide coenzymes reached steady-state levels in light, NADP$^+$ decreased and NAD$^+$ increased. The changes of nicotinamide coenzymes induced by CMU were quite similar to those observed when the light was turned off in the absence of CMU (Fig. 1). The addition of

TABLE II

EFFECTS OF DSPD AND CCCP ON THE LIGHT-INDUCED CONVERSION OF NAD^+ TO $NADP^+$ AND PHOTOSYNTHETIC $^{14}CO_2$ FIXATION

0.1 ml of the inhibitor solution was added to a test tube containing 0.5 ml of the algal suspension and each test tube was shaken in the dark in the transparent water bath kept at 25°C. After 30 min, 0.03 ml $NaH^{14}CO_3$ solution (88.5 mM) was added and the test tubes were illuminated from below by a bank of day-light fluorescent lamps. The amounts of ^{14}C fixed and of nicotinamide coenzymes were determined after another 5 min. The algal cells suspended in 50 mM Mes-KOH solution, pH 6.5 and 50 mM Hepes-KOH solution (pH 7.6) were used for the experiments with CCCP and DSPD, respectively.

Inhibitor	Concentration (M)	$^{14}CO_2$ fixed (μmol/ml pcv per 5 min)	Δ[$NADP^+$ + NADPH] (nmol/ml pcv per 5 min)
DSPD	0	33.3 (100.0%)	26.7 (100.0%)
	5×10^{-6}	26.6 (80.0%)	21.8 (81.6%)
	5×10^{-5}	19.1 (57.4%)	15.6 (58.4%)
	5×10^{-4}	0.3 (0.8%)	−1.5 (- - - - -)
CCCP	0	30.8 (100.0%)	33.0 (100.0%)
	5×10^{-6}	27.7 (89.9%)	29.5 (89.4%)
	4×10^{-5}	0.2 (0.7%)	−4.7 (- - - - -)

CMU under anaerobic conditions in light caused essentially the same changes (data not shown). These results indicate that the light-induced conversion of NAD^+ to $NADP^+$ requires the operation of photosynthetic electron transport.

Effect of DSPD and CCCP on the light-induced conversion of NAD^+ to $NADP^+$

As shown in Table II, DSPD and CCCP inhibited both the light-induced conversion reaction and CO_2 fixation to the same extent, depending on their concentrations.

Effect of light on the nicotinamide coenzyme levels of chlorophyll-less mutant of Chlorella

Illumination of chlorophyll-less yellow mutant, *C. vulgaris* 211-11 h/20 cells which had been kept in the dark for 30 min in air did not cause appreciable change in either NAD^+-plus-NADH or $NADP^+$-plus-NADPH levels, although a small temporary decrease in NAD^+ and $NADP^+$ and increase in NADPH were observed (data not shown).

Induction of CO_2 fixation and the conversion reaction of NAD^+ to $NADP^+$

A lag-time in photosynthetic O_2 evolution and CO_2 fixation is commonly observed in both algae and higher plant leaves when they are illuminated after a period of darkness [12]. The light-induced conversion of NAD^+ to $NADP^+$ is a reaction which also can be observed after dark preincubation. To examine the

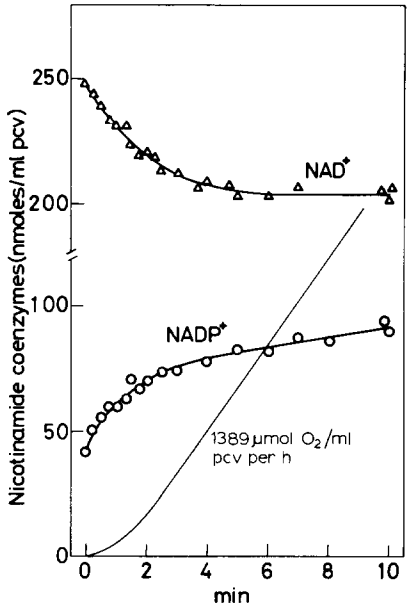

Fig. 6. Simultaneous measurements of CO_2 fixation and the changes in nicotinamide coenzymes. Two oxygen electrode vessels containing 5.9 ml of *Chlorella* cells (20 ml packed cell volume/l) were illuminated at 102 000 erg/cm^2 per s after 30 min dark preincubation. Prior to illumination, 0.1 ml 420 mM $NaHCO_3$ was added.

relationship between the induction of O_2 evolution and the conversion reaction, both were measured under similar conditions (Fig. 6). *Chlorella* cell suspensions which were filled in two oxygen electrode vessels were illuminated at the same time and at the same light intensity. One was used for monitoring O_2 evolution and the other for the assay of nicotinamide coenzymes. The decrease in NAD^+ and the increase in $NADP^+$ started immediately after illumination, and almost half of the conversion of NAD^+ to $NADP^+$ was achieved during the induction period of O_2 evolution, which continued for about 1 min.

Discussion

The light-induced conversion of NAD^+ to $NADP^+$ occurred in the presence and absence of CO_2 under both aerobic and anaerobic atmosphere. In *Chlorella* cells, the light-induced increase in $NADP^+$ was much greater than that in NADPH (Fig. 1 and also Ref. 1). Generally, attention has been paid to the reduction in $NADP^+$ rather than its increase. Using intact chloroplasts isolated from spinach leaves, Lendzian and Bassham [13] have reported a rapid increase in the NADPH/$NADP^+$ ratios upon illumination. Since the total amount of NADPH plus $NADP^+$ remained unchanged, they concluded that light-induced conversion of NAD^+ to $NADP^+$ does not occur in spinach chloroplasts. However, recent studies [14] revealed that the chloroplasts isolated in the dark from spinach leaves which had been preincubated in darkness overnight can carry out this conversion. No such conversion was observed without dark preincubation. In the case of the dark-treated spinach chloroplasts, $NADP^+$ rapidly decreased upon illumination and then increased gradually, while NADPH rapidly increased at first and then remained at a constant level. As a result, the NADPH/$NADP^+$ ratio rose quickly upon illumination. In contrast, no light-induced shift in NADPH/$NADP^+$ ratio was observed in the dark-treated wheat chloroplasts or other plant leaves. Upon illumination, the conversion of NAD^+ to $NADP^+$ was observed in intact leaves of wheat and pea following incubation in the dark overnight. This conversion was also observed in mesophyll protoplasts of wheat leaves which had been kept in the dark for 1 h. The chloroplasts isolated from wheat protoplasts prepared in the dark carried out this conversion. However, the increase in NADPH was relatively slow in all the above cases. These results [14] indicate that the light-induced conversion of NAD^+ to $NADP^+$ generally occurs in algae and higher plants which have been preincubated in the dark, while the pronounced light-induced reduction of $NADP^+$ is observed only in spinach chloroplasts. The reason for this is not clear at this moment.

In this connection, it should be borne in mind that the algal cells were killed with either boiling alkali or acid according to the conventional method [1,15]. Since the turnover rate of NADPH during steady-state photosynthesis should be high [16] and *Chlorella* cells are more resistant to the above treatments than isolated chloroplasts, it can not be sure that the levels of each nicotinamide coenzyme determined in the present experiments reflect the actual redox state, although the reproducible changes were observed after repeated experiments. The effects of the killing procedures on the redox state of nicotinamide coenenzymes were not tested, since the focus of the present study was placed on the conversion of NAD^+ to $NADP^+$.

Inhibition of the conversion by CMU, DSPD and CCCP strongly suggests that it is associated with photophosphorylation, which will supply ATP needed for this process. The fact that the conversion of NAD^+ to $NADP^+$ did not occur under the conditions in which only cyclic photophosphorylation can take place (Fig. 4) further indicates that, in addition to the supply of ATP, electron transport must be operating for the conversion of NAD^+ to $NADP^+$.

Since NAD kinase is the only enzyme known to catalyze the phosphorylation of NAD^+ to $NADP^+$, this enzyme may be responsible for the light-induced conversion of NAD^+ to $NADP^+$. We found that NAD kinase of wheat and pea leaves was rapidly activated by light [14]. Phytochrome-mediated activation of the enzyme was also reported with etiolated higher plants [17,18]. The possibility of this type of activation is excluded in *Chlorella,* since the conversion of NAD^+ to $NADP^+$ is inhibited by both CMU and CCCP, and its dependency on the wavelengths of incident light is similar to that of photosynthesis. It is possible that NAD kinase which is activated via the electron transport system of photosynthesis catalyzes the conversion of NAD^+ to $NADP^+$. The observation that this conversion reaction is susceptible to CMU is

in accord with this inference. It has been shown that the NAD kinase of various C_3 plant leaves is localized in the chloroplasts [14]. We therefore assumed that the light-induced conversion of NAD^+ to $NADP^+$ occurs in the chloroplasts and is catalyzed by photoactivated NAD kinase using photochemically produced ATP.

The conversion of NAD^+ to $NADP^+$ was most active during the induction period of photosynthesis (Fig. 6). Induction of photosynthesis has been attributed to the time needed for the light activation of photosynthetic enzymes as well as for the autocatalytic build up of the photosynthetic intermediates for the reductive pentose phosphate pathway which were depleted during the dark pretreatment [12]. We may assume that the elevation of $NADP^+$ level by the conversion reaction and the subsequent reduction also contribute to the enhancement of the rate of photosynthesis during the induction period.

It has been reported that there are NAD^+-dependent and $NADP^+$-dependent activities of glyceraldehyde-3-phosphate dehydrogenase in the chloroplasts (see, for example, Ref. 19). Since it has been shown that this enzyme is converted to an $NADP^+$-dependent form in the presence of $NADP^+$ and that this conversion is inhibited by NAD^+ [20], the ratio of $NADP^+$ to NAD^+ in the chloroplasts could be one of the important factors regulating the coenzyme specificity of this enzyme. Therefore, the light-induced formation of $NADP^+$ from NAD^+ may be responsible for the above-mentioned conversion of the coenzyme specificity, which would be favorable to the shift of the carbohydrate metabolism in the chloroplasts from glycolysis to starch synthesis which occurs upon illumination.

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