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PHOTOREDUCTION OF FERREDOXIN AND ITS USE IN NAD(P)$^+$ REDUCTION BY A SUBCELLULAR PREPARATION FROM THE PHOTOSYNTHETIC BACTERIUM, *CHLOROBIUM THIOSULFATOPHILUM*

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SUMMARY

1. A subcellular system derived entirely from the photosynthetic green sulfur bacterium, *Chlorobium thiosulfatophilum*, reduced NAD$^+$ and NADP$^+$ in light-dependent reactions.

2. The rate of NAD$^+$ reduction was twice that of NADP$^+$.

3. The reduction of NAD(P)$^+$ required chlorophyll-containing particles, ferredoxin, and a soluble protein fraction, which catalyzed a reduction of NAD(P)$^+$ with reduced ferredoxin. The soluble protein fraction could be replaced by purified ferredoxin–NADP reductase from spinach chloroplasts. The chlorophyll-containing particles photoreduced ferredoxin with sodium sulfide or 2-mercaptoethanol as the electron donor.

4. The rate of NAD$^+$ reduction was comparable to the over-all rate of CO_2 assimilation by *C. thiosulfatophilum* cells.

INTRODUCTION

Reduced nicotinamide–adenine dinucleotides are needed for carbon assimilation in bacterial and plant photosynthesis. In isolated spinach chloroplasts, the photoreduction of NADP$^+$ was shown to occur in three steps[1]: (a) photochemical reduction of ferredoxin; (b) reoxidation of reduced ferredoxin by a flavoprotein enzyme, ferredoxin–NADP reductase; (c) reoxidation of the reduced ferredoxin-NADP reductase by NADP$^+$. The spinach ferredoxin and ferredoxin–NADP reductase necessary for this sequence of reactions have been crystallized[2–4].

In cell-free preparations from photosynthetic bacteria (*Chromatium*[5–7] and *Chlorobium thiosulfatophilum*[6]) the reduction of nicotinamide–adenine dinucleotides by the H_2–hydrogenase system also requires ferredoxin. Since photosynthetic bacteria (unlike chloroplasts) contain hydrogenase, they could reduce NAD$^+$ and NADP$^+$ with hydrogen gas, independently of light. There was no evidence, however, that in photosynthetic bacteria a photochemical reduction of ferredoxin could be coupled to a production of reduced NAD or NADP.

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The subcellular chlorophyll-containing particles (henceforth called particles) from *C. thiosulfatophilum* have been found to reduce ferredoxin photochemically[8,9]. The photoreduction of ferredoxin was coupled to pyruvate[8] and α-ketoglutarate[9] synthesis by two new ferredoxin-dependent reductive carboxylation reactions that are key steps in the reductive carboxylic acid cycle of bacterial photosynthesis[10,11]. The present results provide evidence for a role for photoreduced ferredoxin in the reduction of NAD^+ and to a lesser degree, $NADP^+$ by subcellular preparations from *C. thiosulfatophilum*. The reaction requires bacterial particles to generate photochemically reduced ferredoxin (with an electron donor such as sodium sulfide or 2-mercaptoethanol) and a soluble enzyme fraction to catalyze the reduction of NAD^+ or $NADP^+$ by reduced ferredoxin.

METHODS

C. thiosulfatophilum was grown as described previously[8]. Chlorophyll-containing particles were prepared[8] from cells sonicated in 0.05 M potassium phosphate buffer (pH 6.5) and were washed twice with this buffer prior to use. Particles prepared in this way photoreduced ferredoxin reproducibly (determined by either CO_2 fixation[8,9] or NAD^+ reduction). However, all attempts to obtain significant rates of photophosphorylation with these preparations were unsuccessful.

The soluble protein fraction was prepared from 50 g freshly harvested *C. thiosulfatophilum* cells suspended in 100 ml 0.02 M potassium phosphate buffer (pH 7.4) with a Waring blendor. The cell suspension was sonicated 6 min with a Branson sonifier (power setting, 8) and was centrifuged 10 min at 39000 × *g*. The precipitate was discarded and, to remove ferredoxin, the supernatant fraction was passed through a 3 cm × 3 cm DEAE-cellulose column, which was equilibrated with 0.02 M potassium phosphate buffer (pH 7.4). The DEAE-cellulose treated extract was centrifuged 1 h in a Spinco preparative ultracentrifuge (105000 × *g* maximum force). The precipitate was discarded and the supernatant fraction, containing the active enzyme, was lyophilized overnight and stored as a dry powder at —20°. 20 mg of the powder was equivalent to 7.5 mg protein, determined by the phenol reagent assay. Ferredoxin-NADP reductase, isolated in pure form from spinach chloroplasts by the procedure of SHIN, TAGAWA AND ARNON[4], was kindly supplied by R. CHAIN.

Protein was estimated by the modified phenol reagent assay of RABINOWITZ AND PRICER[17] using bovine serum albumin as a standard. Chlorobium chlorophyll was determined as described by STANIER AND SMITH[18].

RESULTS AND DISCUSSION

Table I shows the capacity of a subcellular preparation from *C. thiosulfatophilum* to reduce NAD^+ and $NADP^+$. Both dinucleotides were reduced in a light-dependent reaction by this preparation but the rate of NAD^+ reduction was twice that of $NADP^+$. In several other experiments, in which the ratio of particles to soluble fraction was varied, the rate of reduction of NAD^+ was consistently about twice that of $NADP^+$. Because of its more rapid rate of reduction, only NAD^+ reduction was measured in subsequent experiments.

Table II shows the requirements for the photoreduction of NAD^+. The reaction

TABLE I

REDUCTION OF NAD$^+$ AND NADP$^+$ BY A SUBCELLULAR PREPARATION FROM *C. thiosulfatophilum*

The complete system contained: chlorophyll-containing particles from *C. thiosulfatophilum* equivalent to 3 mg Chlorobium chlorophyll; *Chromatium* ferredoxin, 200 μg; a soluble protein fraction from *C. thiosulfatophilum*, 7.5 mg protein; and the following in μmoles: Tris buffer (pH 8.5), 300; 2-mercaptoethanol, 25; NAD$^+$ or NADP$^+$, 10. Final vol., 3.0 ml. Gas phase, argon. The reaction was carried out at 20° in Warburg vessels for 90 min under illumination (10000 lux) as indicated and was stopped by adding 6 ml neutralized saturated $(NH_4)_2SO_4$ solution[7]. The precipitated protein was centrifuged off and the supernatant fraction was analyzed for NADH and NADPH by measuring absorbance at 340 mμ.

Treatment	*NAD(P)$^+$ reduced (μmoles)*
NAD$^+$, dark	0.06
NAD$^+$, light	1.38
NADP$^+$, dark	0.10
NADP$^+$, light	0.62

TABLE II

REQUIREMENTS FOR THE REDUCTION OF NAD$^+$ BY A SUBCELLULAR SYSTEM FROM *C. thiosulfatophilum*

The complete system contained: chlorophyll-containing particles from *C. thiosulfatophilum* equivalent to 3 mg Chlorobium chlorophyll; *Chromatium* ferredoxin, 200 μg; soluble protein fraction from *C. thiosulfatophilum*, 7.5 mg protein; crystalline lactate dehydrogenase, 0.1 mg; and the following in μmoles: Tris buffer (pH 8.5), 300; 2-mercaptoethanol, 25; NAD$^+$, 2; sodium pyruvate, 10. Final vol., 3.0 ml. Gas phase, argon. The reaction was carried out at 20° in Warburg vessels for 90 min under illumination (10000 lux) and was stopped and analyzed for lactate as described by HOHORST[12].

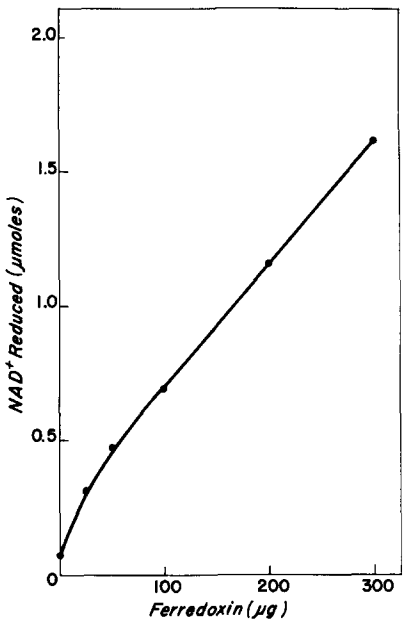
Treatment	*NAD$^+$ reduced (μmoles)*
Light	
Complete	1.3
Ferredoxin omitted	0.3
2-Mercaptoethanol omitted*	0.2
Particles omitted	0.2
Soluble fraction omitted	0.1
NAD$^+$ omitted	0.05
Dark	
Complete	0.2

* In a parallel experiment, the NAD$^+$ reduced (light dependent) with 25 μmoles Na_2S as electron donor was 105% that observed with 2-mercaptoethanol.

was dependent on reduced ferredoxin and an electron donor (Na_2S or 2-mercaptoethanol). The reduction of NAD$^+$ was also dependent on the particles, which catalyzed the photoreduction of ferredoxin, and on the soluble fraction, which contained an enzyme that catalyzes the reduction of NAD$^+$ by reduced ferredoxin. Although a detailed characterization of this enzyme must await its purification, it appears to be the bacterial analog of ferredoxin–NADP reductase[2,3,1] from spinach chloroplasts. However, unlike the chloroplast enzyme, which is bound to grana and is more active with NADP$^+$ than NAD$^+$, the *C. thiosulfatophilum* enzyme is soluble and, at least at

the present level of purity, reduces NAD^+ more rapidly than $NADP^+$. The soluble nature of this enzyme[6] and of a similar enzyme from *Chromatium*[6,7] was previously found in experiments with ferredoxin reduced by the H_2-hydrogenase system.

Fig. 1 shows the effect of ferredoxin concentration on the reduction of NAD^+. The rate of reduction was proportional to the ferredoxin added up to 50 μg/3 ml, but no saturation with ferredoxin was observed even at the highest level tested (300 μg/3 ml).



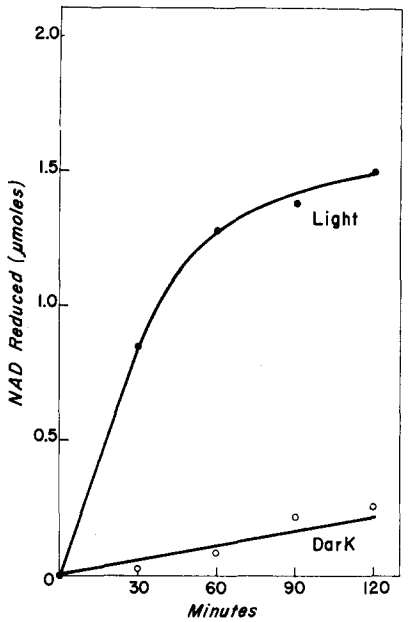


Fig. 1. Effect of ferredoxin concentration on the reduction of NAD^+ by a subcellular preparation from *C. thiosulfatophilum*. Except for varying the ferredoxin concentration, conditions were as described for Table II.

Fig. 2. Time course of light-dependent NAD^+ reduction by a subcellular preparation from *C. thiosulfatophilum*. Except for varying the reaction time, conditions were as described for Table II.

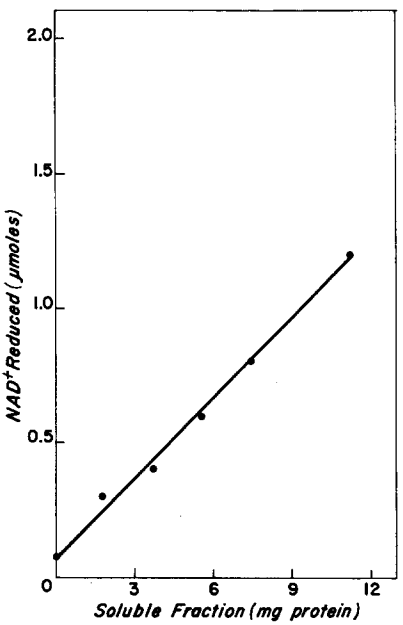
The ferredoxin used in these experiments was isolated from *Chromatium*. Native *C. thiosulfatophilum* ferredoxin was also active in reducing NAD^+, but its instability (*cf.* ref. 8) prevented its routine use. The ability of the *C. thiosulfatophilum* particles to photoreduce ferredoxin from *Chromatium* (and *Clostridium pasteurianum*) was observed previously when photoreduced ferredoxin was used for CO_2 fixation *via* pyruvate synthase[8] and α-ketoglutarate synthase[9].

Fig. 2 shows the time course and the light dependence of the reduction of NAD^+ by the subcellular preparation from *C. thiosulfatophilum*. The light-dependent reaction was linear for at least 30 min. The rate of NAD^+ reduction during the first 30 min was 0.6 μmole NAD^+ reduced/h per mg Chlorobium chlorophyll.

Fig. 3 shows the effect of the concentration of the soluble protein and Fig. 4 shows the effect of the concentration of Chlorobium chlorophyll. NAD^+ reduction was proportional to the soluble protein added up to at least 11 mg/3 ml (Fig. 3). The rate of photoreduction of NAD^+ increased with increasing chlorophyll (up to at least

12 mg/3 ml) but the response was nonlinear at levels greater than 1.5 mg chlorophyll per 3 ml (Fig. 4).

The data presented so far support the idea of a noncyclic electron flow mechanism in *C. thiosulfatophilum*, as described by ARNON *et al.*[19] for *Chromatium* cells and by NOZAKI, TAGAWA AND ARNON[20,21] for particles (chromatophores) from *Rhodospirillum rubrum*. However, our attempts to obtain a light-induced formation of ATP with the *C. thiosulfatophilum* system under either noncyclic conditions (in the presence of NAD+



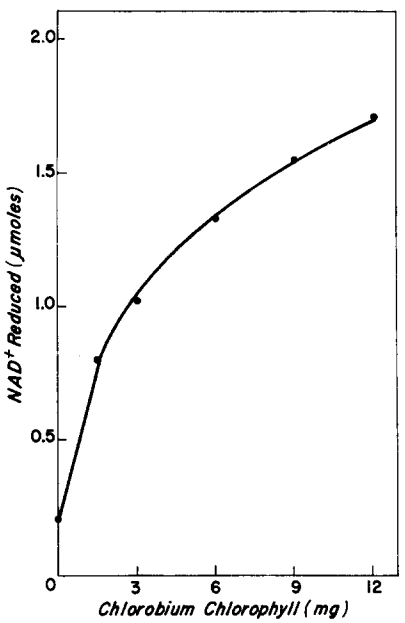


Fig. 3. Effect of protein concentration on the reduction of NAD+ by a subcellular preparation from *C. thiosulfatophilum*. Except for varying concentration of the soluble fraction from *C. thiosulfatophilum*, conditions were as described for Table II.

Fig. 4. Effect of chlorophyll concentration on the reduction of NAD+ by a subcellular preparation from *C. thiosulfatophilum*. Except for varying the Chlorobium chlorophyll concentration, conditions were as described for Table II.

as described above) or cyclic conditions (in the presence or absence of cofactors such as phenazine methosulfate or vitamin K_3) have so far been unsuccessful. Since the *C. thiosulfatophilum* particles do not catalyze photophosphorylation under our experimental conditions, the present results cannot be explained by a reversed electron flow mechanism, as proposed for *R. rubrum* by KEISTER AND YIKE[13,14] which is strictly dependent on a light-induced formation of ATP (or of a hypothetical high-energy intermediate which they believe to be a precursor of ATP).

Instead, the reduction of NAD+ by a subcellular preparation from *C. thiosulfatophilum* appears to follow the sequence of reactions established for the reduction of NADP+ by spinach chloroplasts[1]. The similarity between the chloroplast and the *C. thiosulfatophilum* systems is strengthened by the results shown in Table III. Here the bacterial "soluble fraction" was replaced by ferredoxin–NADP reductase[2,3,1]

TABLE III

REDUCTION OF $NADP^+$ BY PARTICLES FROM *C. thiosulfatophilum* AND THE FERREDOXIN–NADP REDUCTASE FROM SPINACH CHLOROPLASTS

Other than omitting the soluble protein fraction from *C. thiosulfatophilum* and adding 0.1 mg pure ferredoxin–NADP reductase from spinach chloroplasts[4], conditions were as described for the $NADP^+$ treatments of Table I.

Treatment	*$NADP^+$ reduced (μmoles)*
Light	
Complete	2.9
Ferredoxin omitted	0.5
2-Mercaptoethanol omitted	0.4
Dark	
Complete	0.3

from spinach chloroplasts. Since the chloroplast enzyme reduced $NADP^+$ more rapidly than NAD^+, $NADP^+$ was used as the terminal electron acceptor in this experiment. Table III shows that significant amounts of $NADP^+$ were reduced only in the light and only in the presence of ferredoxin. An electron donor such as 2-mercaptoethanol was also required.

The present findings show that in a subcellular system from *C. thiosulfatophilum* the photoreduction of ferredoxin is coupled to a reduction of NAD^+ and, less effectively, $NADP^+$. The maximal reduction rate observed was 0.6 μmole NAD^+ reduced/h per mg Chlorobium chlorophyll. This value, which was obtained with a cell-free system, is comparable to the rate of CO_2 assimilation by whole cells of *C. thiosulfatophilum*[10] (in five experiments, the rate of CO_2 assimilation ranged from 0.7 to 2.4 μmoles CO_2 assimilated per h per mg Chlorobium chlorophyll), and supports the view that photoreduced ferredoxin provides electrons necessary to form the NAD(P)H needed by the cell.

As in chloroplasts, the reduction of NAD^+ by *C. thiosulfatophilum* preparations required chlorophyll-containing particles, which catalyzed a photoreduction of ferredoxin (in the presence of the physiological electron donor Na_2S or an artificial donor such as 2-mercaptoethanol) and NAD(P) reductase, which catalyzed reduction of $NAD(P)^+$ with reduced ferredoxin (Eqns. 1 and 2). Unlike chloroplasts, the bacterial NAD(P) reductase was soluble; it could also be replaced by a purified ferredoxin-NADP reductase from spinach chloroplasts.

$$\text{Ferredoxin}_{ox} + \text{electron donor} \xrightarrow[h\nu]{\text{particles}} \text{ferredoxin}_{red} + \text{oxidized electron donor} \quad (1)$$

$$2\ \text{Ferredoxin}_{red} + NAD(P)^+ \xrightarrow[\text{protein}]{\text{soluble}} NAD(P)H + 2\ \text{ferredoxin}_{ox} \quad (2)$$

In Eqn. 2, bacterial ferredoxin is represented as a one-electron carrier[2,3]. Other investigators, however, have reported that bacterial ferredoxin is a two-electron carrier[15,16].

NOTE ADDED IN PROOF (Received March 21st, 1969)

After this paper had been sent to press, the work of KLEMME AND SCHLEGEL[22] was brought to our attention. These authors found a dependence on light for the reduction of NAD^+ with molecular hydrogen by chromatophores from *Rhodopseudomonas capsulata*. The relation of this mechanism to the light-dependent, ferredoxin-mediated reduction of $NAD(P)^+$ with hydrogen by subcellular preparations from *Chromatium* described previously (ref. 7) and to the *C. thiosulfatophilum* mechanism described herein remains to be determined.

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