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Ferredoxin-dependent reduction of nicotinamide–adenine dinucleotides with hydrogen gas by subcellular preparations from the photosynthetic bacterium, *Chromatium*

In 1961, ARNON (Table 7 in ref. 1) showed that cell-free extracts from the photosynthetic bacterium, *Chromatium*, reduced NAD^+ and $NADP^+$ with H_2 (independently of light) in the presence of benzyl viologen as an artificial electron carrier. In the same year, K. TAGAWA AND M. NOZAKI (unpublished data from this laboratory) and LOSADA, WHATLEY AND ARNON[2] isolated from *Chromatium* cells the protein now known as ferredoxin[3] (then described as "photosynthetic pyridine nucleotide reductase"). A role for ferredoxin as an electron carrier between an H_2–hydrogenase system and the ferredoxin–NADP reductase[14] of spinach chloroplasts was observed by TAGAWA AND ARNON[4] and by subsequent investigators[5–7] with other enzyme preparations. However, for several years there was no evidence for a role for ferredoxin in the reduction of NAD^+ or $NADP^+$ with H_2 in *Chromatium*.

In 1964, BUCHANAN, BACHOFEN AND ARNON[8] showed that, in extracts of *Chromatium* cells, ferredoxin, reduced in the dark with H_2 and the native hydrogenase, drives the reductive carboxylation of acetyl-CoA to pyruvate. During that investigation, a ferredoxin-dependent reduction of NAD^+ with H_2 was observed with *Chromatium* extracts[8,9] (Eqn. 1).

$$H_2 + NAD^+ \xrightarrow[\text{enzymes}]{\text{ferredoxin}} NADH \qquad (1)$$

BUCHANAN, BACHOFEN AND ARNON found that the addition of NAD^+, although not affecting pyruvate synthesis itself, markedly increased total CO_2 assimilation. They concluded that in their system NAD^+ was first reduced by H_2 and was then used in the reductive reactions that converted newly formed pyruvate to alanine, aspartate, and glutamate—the main early products of photosynthesis in *Chromatium*[10,11].

The ability of *Chromatium* extracts to reduce nicotinamide–adenine dinucleotides with H_2 in a ferredoxin-dependent reaction was confirmed in 1965 by WEAVER, TINKER AND VALENTINE[12], who also described ferredoxin-dependent NAD^+ reduction in extracts of another photosynthetic bacterium, *Chlorobium thiosulfatophilum*. WEAVER, TINKER AND VALENTINE separated *Chromatium* extracts into the soluble and particulate components and found that only the soluble fraction was needed for H_2-linked NAD^+ reduction. The particulate fraction had no effect on the reaction.

This communication describes the requirements for the reduction of NAD^+ and $NADP^+$ by a *Chromatium* cell-free extract and by its soluble and particulate components. The experiments reported here were carried out without illumination, with H_2 as the reductant. So far it has not been possible with these preparations to reduce NAD^+ or $NADP^+$ photochemically or to replace H_2 with other reductants.

Fig. 1 shows the time course of H_2 uptake with NAD^+ and $NADP^+$ by a cell-free extract of *Chromatium* that had been passed through a DEAE-cellulose column to remove ferredoxin. H_2 uptake, observed with either NAD^+ or $NADP^+$, proceeded linearly for a least 60 min. The rate of reaction was 4 times greater with NAD^+ than

with $NADP^+$. The higher activity of NAD^+ in this reaction is consistent with the earlier findings of ARNON[1] and WEAVER, TINKER AND VALENTINE[12].

Fig. 1 also shows that with either NAD^+ or $NADP^+$, H_2 uptake was strictly dependent on ferredoxin. As in the *Chromatium* pyruvate synthase system[8], the native ferredoxin and ferredoxin from the nonphotosynthetic bacterium, *Chlostridium pasteurianum*, showed approximately the same activity in the reduction of NAD^+ and $NADP^+$ with H_2. Because of its greater abundance and ease of purification, ferredoxin from *C. pasteurianum* rather than *Chromatium* was used in all subsequent experiments.

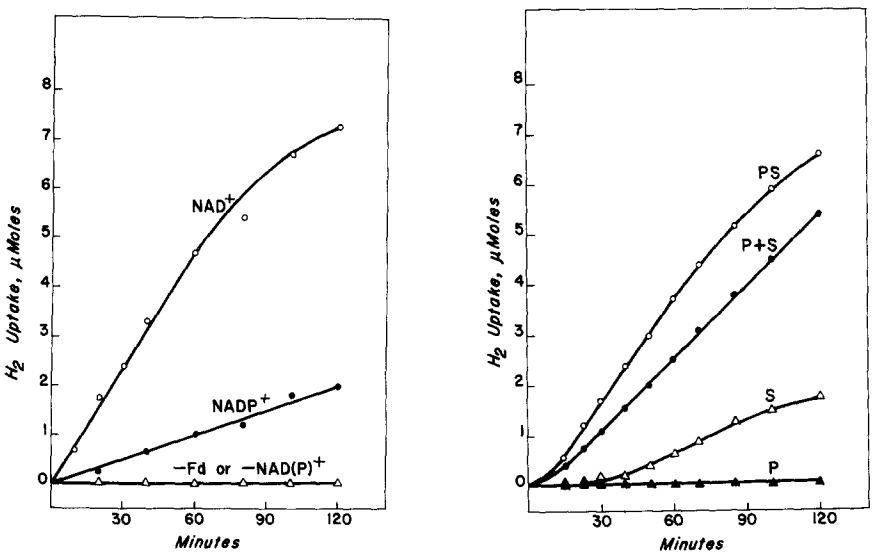

Fig. 1. Effect of ferredoxin (Fd) and $NAD(P)^+$ on H_2 uptake by a cell-free extract from *Chromatium*. The complete system contained a cell-free extract (containing 2.5 mg chlorophyll and 25 mg protein, see below) from *Chromatium*, 200 μg ferredoxin from *C. pasteurianum*, and the following in μmoles: potassium phosphate buffer (pH 6.5), 200; NAD^+ or $NADP^+$, 10; and $MnCl_2$, 4. Final vol., 3.0 ml. Gas phase was H_2. The reaction was carried out at 30° in Warburg flasks, containing 0.1 ml 20% KOH in the center well, and was initiated by NAD^+ or $NADP^+$ added from a side-arm to the main compartment. The uptake of H_2 was measured manometrically. The cell-free extract was prepared from *Chromatium* cells and was treated with DEAE-cellulose as described previously[8]. Methods for growing the cells, estimating protein and *Chromatium* chlorophyll, and isolating ferredoxin were also as previously given[8].

Fig. 2. H_2 uptake by soluble and particulate fractions of cell-free *Chromatium* extracts. A cell-free extract (PS, containing 2.5 mg chlorophyll and 25 mg protein) from *Chromatium* or its soluble (S) and particulate (P) components at equivalent levels was added as indicated. The S and P fractions were obtained by centrifuging the extract for 90 min at 105000 × *g* in a Spinco preparative ultracentrifuge. Other conditions were as described in Fig. 1.

Fig. 2 shows the time course of H_2 uptake by a cell-free extract (PS) and by its soluble (S) and particulate (P) components in the presence of NAD^+ and ferredoxin. About 25% of the activity of the cell-free extract was recovered in the soluble fraction; the activity recovered in the particulate fraction alone, which contained the "chromatophores", was negligible. However, a recombination of the particulate and soluble fractions gave an almost complete restoration of the reduction activity of the original cell-free extract. The requirement for both fractions for maximal NAD^+

TABLE I

STOICHIOMETRY BETWEEN H_2 UPTAKE AND NAD^+ REDUCTION BY SUBCELLULAR FRACTIONS FROM *Chromatium*

A cell-free extract (PS) and a reconstituted extract (P + S) from *Chromatium* were added as indicated. Reaction time was 60 min. The reaction was stopped by adding 6 ml neutralized, satd. $(NH_4)_2SO_4$ solution[1], precipitated protein was centrifuged off, and the supernatant fraction was analyzed for NADH by measuring absorbance at 340 mμ. Other conditions were as given in Figs. 1 and 2.

Fraction	*H_2 uptake (μmoles)*	*NAD^+ reduced (μmoles)*
Cell-free extract (PS)	4.7	3.5
Reconstituted extract (P + S)	4.1	3.7

TABLE II

NAD^+ REDUCTION WITH H_2 BY THE SOLUBLE (S) AND PARTICULATE (P) FRACTIONS FROM *Chromatium*

Ferredoxin and benzyl viologen (0.1 μmole) were added to the particulate (P) and soluble (S) fractions as indicated. The reaction was stopped and analyzed for NADH as given in Table I. Other conditions were as given in Figs. 1 and 2.

Treatment	*NAD^+ reduced (μmoles)*
P	0.7
P + ferredoxin	1.5
P + benzyl viologen	2.8
S	1.2
S + ferredoxin	2.8
S + benzyl viologen	9.6
P + S	1.5
P + S + ferredoxin	8.4
P + S + benzyl viologen	8.8

reduction activity does not agree with the results of WEAVER, TINKER AND VALENTINE[12] who found activity exclusively in the soluble fraction.

Table I shows that with either the original or the reconstituted cell extract the molar ratio between H_2 uptake and the NAD^+ reduced was approx. 1:1 (see Eqn. 1).

Although both the soluble and particulate components were required for maximal NAD^+ reduction activity with H_2 when ferredoxin was added as the electron carrier, the soluble fraction alone gave maximal activity when an artificial electron carrier, benzyl viologen, replaced ferredoxin (Table II). The activity of the particles alone with benzyl viologen was low. The high rates of reduction of NAD^+ by the soluble fraction with H_2 in the presence of benzyl viologen indicate that both hydrogenase and an NAD^+ reductase are present in the soluble fraction. The presence of both enzymes in the soluble fraction was also established by independent enzyme assays. The NAD^+ reductase was assayed by following its NADH–dichlorophenolindophenol diaphorase activity[13,14] and hydrogenase was assayed colorimetrically following the reduction of benzyl viologen with H_2*. However, hydrogenase was

* B. B. BUCHANAN, unpublished procedure.

present not only in the soluble fraction but was also bound to the particles. Based on the benzyl viologen assay, hydrogenase activity was approximately equally distributed between the soluble and particulate fractions.

We conclude that cell-free extracts of *Chromatium* can reduce NAD^+ and, to a lesser degree, $NADP^+$ with H_2 as the reductant. For maximal activity the reaction depends on ferredoxin and on both the soluble and particulate fractions of the cell extract. Since both hydrogenase and NAD^+ reductase are in the soluble fraction, the contribution of the particles to NAD^+ reduction is not clear. It is possible that the particles contain an unknown bound component which links hydrogenase to ferredoxin or, alternatively, that only a bound hydrogenase, and not a soluble hydrogenase, can couple efficiently to ferredoxin in the utilization of H_2.

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Light-absorption and light-scattering changes during shrinking and swelling of chloroplasts

It is now established that isolated chloroplasts undergo either shrinkage[1,2] or swelling[3,4] upon illumination. The direction of the volume change depended on the nature of the suspending medium; chloroplasts suspended in a medium containing

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $TMQH_2$, trimethyl-1,4-benzoquinol; DCIP, 2,6-dichlorophenolindophenol.