BBA 66047

# SOLUBILIZATION AND PROPERTIES OF THE HYDROGENASE OF *CHROMATIUM**

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(Received October 6th, 1969)

## SUMMARY

Cell-free extracts of *Chromatium* were separated into a soluble and a particulate chromatophore fraction and the latter fraction contained most of the hydrogenase activity of the cells as well as the ferredoxin-dependent $H_2$–$NAD^+$ reducing activity. The particulate hydrogenase was solubilized by treating either cells or particles with Triton X-100 or deoxycholate. The solubilized hydrogenase prepared from cells could reduce $NAD^+$ in the presence of ferredoxin while the solubilized hydrogenase prepared from particles could not. Benzyl viologen stimulated $NAD^+$ reduction in all preparations. The hydrogenase of *Chromatium* activates $H_2$ by a heterolytic split to form an enzyme hydride.

## INTRODUCTION

The reduction of pyridine nucleotides by reduced ferredoxin occurs in all photosynthetic organisms and leads to the reductive assimilation of carbon. In organisms containing hydrogenase, $H_2$ can serve as the primary reducing agent by reducing the pyridine nucleotides in the dark as well as in the light. This reaction has been studied in many microorganisms both photosynthetic and non-photosynthetic[1–8], and various factors, including ferredoxin and FAD, have been implicated in the transfer of hydrogen from $H_2$ to pyridine nucleotides.

BUCHANAN *et al.*[9], in 1964, reported indirect evidence suggesting that extracts of *Chromatium* require ferredoxin for the reduction of $NAD^+$ by $H_2$. In 1965, WEAVER *et al.*[10] showed directly that ferredoxin is essential for the reduction of pyridine nucleotides by $H_2$ in extracts of *Chromatium* and reported that the $H_2$–$NAD^+$ reducing system is in the soluble portion of the extract with no activity in the particulate chromatophore fraction. Recently, BUCHANAN AND BACHOFEN[11] confirmed the ferred-

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oxin requirement for $NAD^+$ reduction in *Chromatium* but reported that only 25% of the activity of the extract was recovered in the soluble fraction with negligible activity in the particulate fraction. Recombination of the two fractions gave the original activity of the extract. Both the soluble and particulate fractions contained equal hydrogenase activity as measured by benzyl viologen reduction by $H_2$.

The present report is a study of the properties of the hydrogenase of *Chromatium*. Most of the hydrogenase of this organism was found to be particulate and this fraction contained the ferredoxin-dependent $H_2$–$NAD^+$ reducing system with very little activity in the soluble fraction. The particulate hydrogenase was solubilized and its properties investigated.

## MATERIALS AND METHODS

### *Preparation of cell extracts*

Samples of *Chromatium*, Strain D, were obtained from four different laboratories. All contained an aerobic contaminant which gave white colonies when grown aerobically on nutrient agar plates. By serial dilutions and anaerobic growth in deep agar tubes, individual colonies of pure *Chromatium* were isolated and used for the present investigation.

Cells were grown in a water bath at 30° illuminated by two banks of 75-W incandescent lamps which gave a light intensity of 300 foot candles. The cultures were maintained on a bicarbonate medium[12] in flasks which were filled to the neck and tightly stoppered. Cells were transferred to new media every 48 h (20% inoculum) and after three transfers in bicarbonate medium, a 30% inoculum was made into large volumes of malate medium[12]. After 48-h growth in this medium, the cells were collected by centrifugation and washed 3 times with distilled water. The cells were stored at −20° either as a frozen paste or suspended in a minimum volume of 0.05 M phosphate buffer (pH 7.2) containing 0.01 M mercaptoethanol. The hydrogenase content of the cells was greater when grown on malate medium than when grown on bicarbonate medium.

Cell-free extracts of *Chromatium* were prepared either by sonic vibration of cell suspensions in a 9 kcycle Raytheon oscillator for 30 min at 2°, or by passing a cell suspension through a French pressure cell at 20 000 lb/inch$^2$. Extracts prepared by the latter method were considerably more viscous than those prepared by sonic vibration and were incubated for 1 h at 0° with deoxyribonuclease (5 $\mu$g/ml of extract) to decrease the viscosity. The disrupted cells were centrifuged at 14 000 × $g$ for 20 min to remove unbroken cells and cell debris.

Centrifugation of the cell-free extract for 90 min at 100 000 × $g$ yielded a supernatant soluble fraction and a particulate chromatophore fraction. The particulate fraction was washed by suspension in 0.05 M phosphate buffer (pH 7.2) containing 0.01 M mercaptoethanol, followed by recentrifugation at 100 000 × $g$ for 90 min. The washed particles were suspended in a minimum volume of buffer. Protein was estimated by the method of LOWRY *et al.*[13].

### *Activity assay*

Hydrogenase activity was measured using the tritium exchange assay[14] or the reduction of benzyl viologen with $H_2$ manometrically. In the exchange assay, the

flask contained 0.5 ml of $^3H_2O$ (5 mC), the sample to be assayed, 0.5 ml of 1.5 M phosphate buffer (pH 6.7) and enough distilled water to make the final volume 4.5 ml. The flasks were cooled in ice water for 15 min and then 15 mg of $Na_2S_2O_4$ in 0.5 ml of 0.15 M phosphate buffer (pH 6.7) was added. After evacuation on a high vacuum manifold, the flasks were filled with prepurified $H_2$. The evacuation procedure was repeated 3 times over a period of 10 min. After the final filling with $H_2$, the flasks were shaken at 25° and gas samples removed at intervals to be analyzed for their tritium content in an ionization chamber connected to a vibrating reed electrometer[14].

The manometric assays for benzyl viologen or $NAD^+$ reduction were performed in a Warburg apparatus in the convention manner[15] by measuring the rate of $H_2$ uptake. The main compartment of the flask contained enzyme and sufficient 0.15 M phosphate buffer (pH 6.7) to bring the volume to 2.4 ml. One side arm contained 5–10 μmoles of the substrate to be reduced in 0.2 ml of buffer. The center well contained 0.2 ml of FIESER's[16] solution and a strip of fluted filter paper and the second side arm contained an additional 0.2 ml of FIESER's[16] solution to facilitate removal of $O_2$. The flasks were gassed with $O_2$-free $H_2$ for 10 min and shaken at 25° for 3 h before the substrate was tipped in and the rate of $H_2$ uptake measured. When the effect of cofactors on the rate of $NAD^+$ reduction was being studied, the cofactors were added to the main compartment of the flask and allowed to equilibrate with the hydrogenase before the pyridine nucleotides were tipped in from the side arm.

In the tritium exchange assay, a unit of activity is defined as an exchange rate of 1 mV/h. In the manometric assays, a unit of activity is defined as the uptake of $H_2$ at a rate of 1 μl/min.

## RESULTS

### *Pyridine nucleotide reduction in Chromatium cells*

Whole cells of *Chromatium* contain an active hydrogenase as measured by either the tritium exchange assay or the reduction of benzyl viologen with $H_2$. These cells

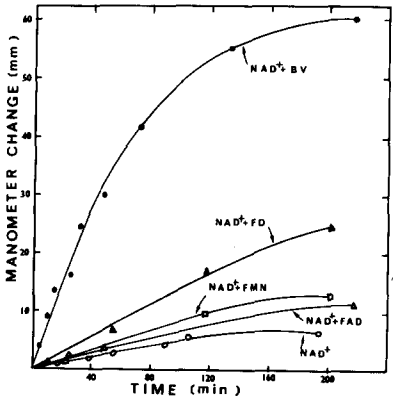


Fig. 1. Effect of cofactors on $NAD^+$ reduction by *Chromatium* cells. $NAD^+$, 5 μmoles, were present in the side arm and the cofactors were in the main compartment. The cofactors were added in the following quantities: FMN, 0.5 μmole; FAD, 0.5 μmole; clostridial ferredoxin (FD), 0.05 μmole, benzyl viologen (BV), 0.5 μmole.

catalyzed a slow reduction of $NAD^+$ by $H_2$ and $NADP^+$ was reduced at half the rate of $NAD^+$ as reported previously[10,11]. The rate of $NAD^+$ reduction by cells was quite low, so the effect of several cofactors was investigated. It can be seen from Fig. 1 that FAD and FMN (0.5 $\mu$mole) had only a slight stimulatory effect. Ferredoxin from *Clostridium pasteurianum* (0.05 $\mu$mole), however, increased the rate of $NAD^+$ reduction about 3-fold. BUCHANAN AND BACHOFEN[11] reported that both clostridial and *Chromatium* ferredoxin had equal activities in this system. Benzyl viologen (0.5 $\mu$mole) increased the rate of $NAD^+$ reduction more than 10-fold. Similar results were obtained for $NADP^+$ reduction.

*Pyridine nucleotide reduction in Chromatium extracts*

In order to further study the hydrogenase and $H_2$–$NAD^+$ reducing system, it was necessary to study these enzymes in cell-free extracts. Accordingly, *Chromatium* cells were disrupted by sonic oscillation and cell-free extracts prepared by centrifugation at 14 000 $\times g$. The extract was then separated into a soluble fraction and a particulate chromatophore fraction by centrifugation at 100 000 $\times g$ and both fractions assayed for hydrogenase and $NAD^+$ reducing activity. The distribution of activities among the two fractions is shown in Table I. It is clear that most of the

TABLE I

HYDROGENASE AND $NAD^+$ REDUCING ACTIVITY OF SOLUBLE AND PARTICULATE FRACTIONS

When used in a hydrogenase assay, 10 $\mu$moles of benzyl viologen were present while 0.5 $\mu$mole was present when it was used as a cofactor. For $NAD^+$ reduction, 5 $\mu$moles were employed as substrate. 0.012 $\mu$mole of clostridial ferredoxin was used as cofactor. The units of activity are defined in the experimental section.

| *Assay* | *Soluble fraction* | | *Particulate fraction* | |
|---|---|---|---|---|
| | *(units/ml)* | *(%)* | *(units/ml)* | *(%)* |
| $^3H$ exchange | 0.29 | 15 | 3.44 | 85 |
| Benzyl viologen reduction | 2.3 | 28 | 11.60 | 72 |
| $NAD^+$ reduction | 0 | 0 | 0.07 | 100 |
| $NAD^+$ + ferredoxin | 0.04 | 6 | 0.72 | 94 |
| $NAD^+$ + benzyl viologen | 0.73 | 40 | 1.00 | 60 |

hydrogenase of *Chromatium*, as measured by tritium exchange or benzyl viologen reduction, is particulate and not soluble. The particles reduced $NAD^+$ very slowly while the soluble portion had no activity. Ferredoxin stimulated $NAD^+$ reduction 10-fold but again most of the activity resided in the particulate fraction. Benzyl viologen also stimulated $NAD^+$ reduction but in this case considerable activity was found in the soluble fraction. Similar results to those in Table I were obtained when the cells were broken by a French press instead of sonic oscillation.

*Solubilization of particulate hydrogenase*

In order to gain more information about the nature of *Chromatium* hydrogenase and the $H_2$–$NAD^+$ reducing system, the solubilization of the particulate hydrogenase was undertaken. Methods involving high pH, nucleases, or proteolytic enzymes were unsuccessful. Lipid reagents, however, were more successful; butanol solubilized

TABLE II

COMPARISON OF TRITON X-100 AND DEOXYCHOLATE EXTRACTION OF CELLS AND PARTICLES

| | % Solubilized* | Specific** activity (units/mg protein) | Benzyl viologen reduction (units/ml) | $^3H$ exchange (units/ml) | Benzyl viologen / Exchange |
|---|---|---|---|---|---|
| Cells | — | — | 47.80 | 3.88 | 12.30 |
| Particles | — | — | 6.08 | 2.14 | 2.85 |
| Triton-extracted cells | 55 | 0.166 | 7.98 | 2.54 | 3.14 |
| Deoxycholate-extracted cells | 20 | 0.255 | 14.20 | 1.86 | 7.64 |
| Triton-extracted particles | 20 | 0.087 | 1.46 | 0.84 | 1.74 |
| Deoxycholate-extracted particles | 3 | 0.193 | 2.30 | 0.63 | 3.65 |

* Based on hydrogenase content ($^3H$ exchange) of cells.
** By the $^3H$ exchange assay.

13% of the activity, digitonin 11% and hog pancreatic lipase (1.3% concentration at 0°) 18%. The most effective solubilizing agent was Triton X-100 (2% concentration at 0°) which solubilized 45% of the particulate hydrogenase with little loss of activity (Table II). Triton X-100 has been reported to be effective in disrupting chloroplasts[17] as well as the pigment system of chromatophores[18]. Deoxycholate extraction of particles yielded hydrogenase of high specific activity but in a low overall yield (Table II).

The solubilization of the particulate hydrogenase could also be achieved by treating whole cells rather than isolated particles with either Triton X-100 or deoxycholate. Sonication of cells in the presence of 2% Triton X-100 solubilized more than 50% of the hydrogenase activity (Table II). Soluble hydrogenase was also prepared from *Chromatium* cells by sonication in 3% deoxycholate followed by incubation at pH 8 at 37° and fractionation with $(NH_4)_2SO_4$ according to the method of KONDO *et al.*[19]. The procedure used in this study omitted trypsin digestion which was found to be unnecessary. The $(NH_4)_2SO_4$ fractionation removes the deoxycholate and serves as a crude fractionation procedure. Though only 20% of the original activity of the cells was recovered (Table II), it was essentially all soluble and yielded hydrogenase of higher specific activity than that obtained by solubilization with Triton X-100. This method was successful in solubilizing the hydrogenase of *Proteus vulgaris*[20].

The solubilization of the hydrogenase from whole cells and particles by both Triton X-100 and deoxycholate are compared in Table II. Triton extraction of cells gives more than twice the total activity of the deoxycholate extraction but the specific activity is almost half. Deoxycholate extraction of particles gives a very low yield though reasonable specific activity, while the Triton extraction of particles gives a higher yield but a lower specific activity. In both procedures, the yield and specific activity are greater when the cells are extracted than when the particles are extracted.

In Table II are also compared the hydrogenase activity as measured by both tritium exchange and benzyl viologen reduction for all four solubilized preparations. It is clear that the ability to reduce benzyl viologen relative to the exchange activity decreases as the hydrogenase is purified. The ratio is higher in cells than in particles and higher in enzyme extracted from cells than in enzyme extracted from particles.

Since the exchange assay is specific for hydrogenase and independent of any cofactors[14], these results suggest that benzyl viologen reduction in *Chromatium* is dependent on other cofactors or enzymes in addition to hydrogenase.

In the previous discussion, the definition of "soluble" enzyme is the activity not sedimented by centrifugation at 100 000 × *g* for 90 min. The failure to sediment at these centrifugal forces was not due to increased viscosity or density of the extracts since the viscosities and densities of the solubilized enzymes were the same as the buffer solution in which they were dissolved. Extensive dialysis of the preparations did not cause any decrease in solubility. However, the possibility still exists that the detergents may bind to the enzyme or particle to form a micelle which holds the enzyme in solution without making it truly soluble.

*$NAD^+$ reduction by solubilized hydrogenase*

The requirements for the $H_2$–$NAD^+$ reducing system in the four solubilized preparations are summarized in Table III. Only the Triton-extracted enzyme from

TABLE III

$NAD^+$ REDUCTION BY SOLUBILIZED HYDROGENASE

5 μmoles of $NAD^+$ were used as substrate and the cofactors were added in the following quantities: benzyl viologen, 0.5 μmole; FAD, 1.0 μmole; clostridial ferredoxin, 0.016 μmole.

| | *$^3H$ exchange (units/ml)* | *Rate of $NAD^+$ reduction (units/ml) in presence of cofactor* | | | | |
|---|---|---|---|---|---|---|
| | | *None* | *Ferredoxin* | *Benzyl viologen* | *FAD* | *FAD + ferredoxin* |
| Cells | 4.85 | 0.27 | 0.52 | 3.40 | — | — |
| Particles | 5.04 | 0.07 | 0.90 | 1.48 | — | — |
| Triton-extracted cells | 2.54 | 0.06 | 0.54 | 2.94 | 0.36 | |
| Deoxycholate-extracted cells | 1.86 | 0 | 0.16 | 2.94 | 0.11 | 0.20 |
| Triton-extracted particles | 0.84 | 0 | 0 | 0.50 | 0.12 | 0.13 |
| Deoxycholate-extracted particles | 0.63 | 0 | 0 | 0.10 | 0.05 | 0.05 |

cells showed any activity in the absence of cofactors. The soluble enzyme prepared from cells by either method was stimulated by ferredoxin whereas the enzyme prepared from the particles could no longer reduce $NAD^+$ even in the presence of ferredoxin. This may be due to the failure to solubilize the flavoprotein–pyridine nucleotide reductase of the particles. Benzyl viologen, however, stimulated $NAD^+$ reduction in all the preparations, though to a greater extent in enzyme prepared from cells. FAD stimulated all solubilized preparations and this effect was not enhanced by ferredoxin beyond the stimulation by ferredoxin alone. This observation would suggest that these two cofactors are stimulating two different, but not sequential, steps in the overall reaction. Spinach ferredoxin had very little effect. In general, the stimulation of $NAD^+$ reduction by different cofactors seems to be determined by the source of the enzyme (cells or particles) rather than by the method used for solubilization.

*Mechanism of $H_2$ activation by Chromatium hydrogenase*

It has been previously suggested[21] that the activation of $H_2$ by hydrogenase is a heterolytic process resulting in the formation of an enzyme hydride and a proton. Evidence for this mechanism was obtained by studying this exchange reaction between $H_2$ and 100% $^2H_2O$ catalyzed by hydrogenase and observing that $^2HH$ was formed at a greater rate than $^2H_2$. When this reaction was studied with *Chromatium* hydrogenase, the ratio of the rate of $^2HH$ to $^2H_2$ formation was 1.6 suggesting that this hydrogenase catalyzes a heterolytic split of $H_2$. This ratio was less than that in *Proteus vulgaris* (5.0) (ref. 22) and Scenedesmus (2.0) (ref. 23), but greater than in *Desulfovibrio desulfuricans* (0.9) (ref. 24). The lower $^2HH$ to $^2H_2$ ratio in some organisms might be interpreted to mean that the enzyme hydride exchanges with the hydrogen of water to some extent.

## DISCUSSION

We have shown that the hydrogenase of *Chromatium* is particulate and have devised procedures for the solubilization of this hydrogenase. The ferredoxin-dependent $H_2$–$NAD^+$ reducing activity of this organism was also shown to be particulate and this result disagrees with that of WEAVER *et al.*[10] and BUCHANAN AND BACHOFEN[11], who reported that the ferredoxin-dependent $NAD^+$ reduction by $H_2$ resides in the soluble fraction and not in the particulate chromatophore fraction. The reasons for this descrepancy are not apparent but may be due to different media employed for growing the cells. In the present investigation the cells were grown on a malate medium which gave higher hydrogenase activities compared to cells grown on a bicarbonate medium. The two previous studies[10,11] were carried out on cells grown on the inorganic bicarbonate medium and the relative activity of various electron transport cofactors and enzymes may be dependent on the carbon source in the growth medium. In addition, the procedures used for disrupting the cells were not identical in the different investigations.

In all hydrogenase preparations—soluble, particulate or solubilized—benzyl viologen stimulated the reduction of $NAD^+$ by $H_2$, often more effectively than ferredoxin. PECK AND GEST[25] reported that benzyl viologen stimulated the rate of pyridine nucleotide reduction by $H_2$ in *Escherichia coli* extracts. The ability of benzyl viologen, an unphysiological electron carrier, to stimulate the rate of $NAD^+$ reduction by hydrogenase could be due to a rapid reduction of benzyl viologen by hydrogenase followed by a rapid nonenzymatic reduction of ferredoxin by the reduced benzyl viologen followed, in turn, by the reduction of $NAD^+$ by reduced ferredoxin catalyzed by ferredoxin–pyridine nucleotide reductase[26]. Another possibility is that the ferredoxin-dependent $NAD^+$ reduction by $H_2$ requires another intermediate electron carrier which is replaceable by benzyl viologen. A final possibility is that the benzyl viologen reduced by hydrogenase reduces $NAD^+$ by the specific NADH–benzyl viologen reductase described by NAGAI *et al.*[27]. These authors have reported the presence of this enzyme in *Azotobacter vinelandii* where it catalyzes the reversible transfer of electrons from NADH to benzyl viologen independent of hydrogenase or ferredoxin. We have indirect evidence that this activity is present in *Chromatium* extracts.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from the National Science Foundation (GB-8003) and a contract from the U.S. Atomic Energy Commission (AT (30-1)3957). One of the authors (E.F.) held a N.A.S.A. predoctoral fellowship.

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