BBA 65939

PURIFICATION AND PROPERTIES OF THE LIGHT-ACTIVATED HYDROGENASE OF *PROTEUS VULGARIS*

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(Received March 28th, 1969)

SUMMARY

The light activation of the hydrogenase of *Proteus vulgaris* previously observed in whole cells has been demonstrated in cell-free extracts and in the particulate hydrogenase obtained from such extracts The particulate hydrogenase was solubilized by treating it with deoxycholate at pH 8 0 and was further purified by $(NH_4)_2SO_4$ fractionation, by heating at 60° and by Sephadex chromatography This procedure afforded a 65-fold purification, and all fractions exhibited light activation of the hydrogenase, suggesting that light directly affects the hydrogenase A comparison of the light-activated purified enzyme and the one active in the dark showed that there was no difference in the molecular weight (115 000), in the mechanism of hydrogen activation or in the K_m for hydrogen, while v_{max} was greater for the light-activated enzyme The mechanism of light activation is discussed in terms of these findings.

INTRODUCTION

The hydrogenase activity in whole cells of *Proteus vulgaris* is markedly increased by irradiating the cells with visible or ultraviolet light of 2537 Å (refs 1, 2) This activation is irreversible and persists even after the irradiation is terminated or after the cells are exposed to air and probably directly affects the hydrogenase since it is observed in the absence of hydrogen acceptors The present investigation was undertaken to elucidate the mechanism of the light activation Since the hydrogenase of *P vulgaris* is particulate[3], the enzyme was solubilized and further purified. The purified soluble hydrogenase was activated by light and the light-activated enzyme differed from the one active in the dark only in the value of v_{max}

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MATERIALS AND METHODS

The experimental details were similar to those previously described[1,2] Hydrogenase activity was measured by the deuterium exchange assay[4] with 10% ^2H_2O or the tritium exchange assay[5] with 1 mC/ml of ^3HHO in 0 15 M of phosphate buffer (pH 7 0) at 37° The hydrogenase was deoxygenated by adding sodium hydrosulfite (5 mg/ml)[6], and bovine albumin (0 3 mg/ml) was added to prevent denaturation of the enzyme during the assay 1 unit of hydrogenase activity is defined as the quantity of enzyme necessary to catalyze an exchange rate of 1 mV/h in the tritium exchange assay or 0 51% deuterium per h in the deuterium exchange assay[5]

Light activation was performed with visible light as previously described[1] The light intensity at the reaction flask was 15 000 ft-candles The protein concentration was determined using the procedure of LOWRY *et al* [7] or spectrophotometrically at 280 and 260 mμ (ref 8) Approximate molecular weights were determined using sucrose gradient centrifugation[9] in a linear 5–20% gradient in a 0 007 M phosphate buffer (pH 7 0) with hemoglobin and catalase as markers All fractions obtained during the purification procedure were analyzed using disc gel electrophoresis and were stained for both protein and hydrogenase[10,11]

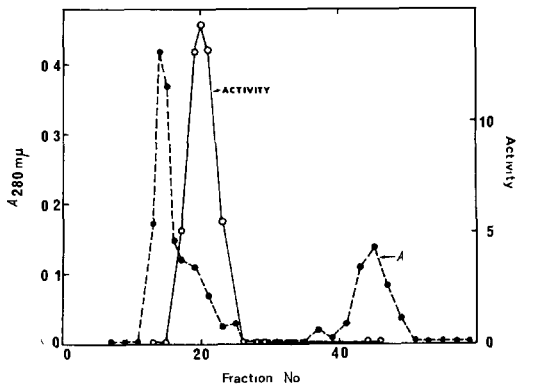
Purification of hydrogenase

P vulgaris cells were grown on a complex medium[4] and were stored frozen as a cell paste until used For the preparation of particulate hydrogenase, cells were suspended in a 0.15 M phosphate buffer (pH 7.0) and were disrupted by sonic oscillation at 10 kcycles for 20 min. The hydrogenase was isolated using differential centrifugation[3]

Soluble hydrogenase was isolated using a modification of the procedure of KONDO *et al* [12]. Cells of *P. vulgaris* were suspended in 3 volumes of 3% sodium deoxycholate in a 0 007 M phosphate buffer (pH 7 0) and were disrupted by sonic oscillation at 10 kcycles for 20 min After removing the cell debris by centrifugation at 12 000 × *g* for 20 min, the supernatant was diluted with 2 vol. of a 0 007 M phosphate buffer (pH 7 0), and the pH of the solution was adjusted to 8 0 by adding 1 M NaOH and then was incubated for 1 h at 37° The pH was readjusted to 7 0 with 1 M HCl before saturated $(NH_4)_2SO_4$ was added to 0 3 saturation The precipitate containing most of the deoxycholate was removed by centrifugation at 12 000 × *g* for 20 min and was discarded The supernatant then was brought to 0 6 saturation with $(NH_4)_2SO_4$ and the precipitate containing the hydrogenase was collected by centrifugation at 12 000 × *g* for 20 min The precipitate was dissolved in a 0 007 M phosphate buffer (pH 7 0) and was centrifuged for 90 min at 100 000 × *g* The supernatant from this high speed centrifugation contained the hydrogenase activity in a soluble form This solubilization procedure recovered 35% of the starting cell-free hydrogenase and had a specific activity of 7 units/mg protein The procedure described by KONDO *et al* [12] utilizes trypsin digestion at pH 8 0 in the presence of deoxycholate to solubilize the hydrogenase We found that trypsin digestion did not increase the yield of soluble hydrogenase from *P vulgaris* over that obtained by incubation at pH 8 0 alone.

Further purification of the soluble hydrogenase was achieved by heating at 60° for 10 min followed by precipitating the enzyme at 0 4 saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in a 0 007 M phosphate buffer (pH 7 0) and was centrifuged at 100 000 × *g* for 90 min The supernatant was concentrated to 4 mg protein

per ml (corresponding to an activity of 70 units/ml) by dialyzing it against 20 volumes of a 30% solution of polyethylene glycol, 20 000 (Fisher Scientific Co) in a 0 007 M phosphate buffer at pH 7 0 The concentrated enzyme solution was chromatographed on a Sephadex G-200 column with a 0 007 M phosphate buffer (pH 7 0) as the eluant The elution pattern of the hydrogenase is shown in Fig 1 This final material was purified 65-fold over the original cell-free extract



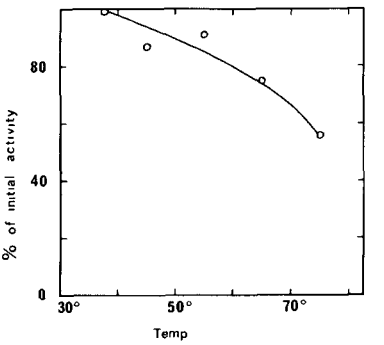


Fig 1 Elution pattern of hydrogenase from Sephadex G-200 The concentrated enzyme solution (4 mg/ml) in a volume of 2 ml was applied to a column (27 5 cm × 2 2 cm) and was eluted with 0 007 M phosphate buffer (pH 7 0) at a flow rate of 10 ml/h 2 5 ml-fractions were collected and assayed for protein (absorbance at 280 mμ) and activity Activity is expressed as mV/h in the tritium exchange assay The void volume, determined with blue dextran, was 33 ml

Fig 2 Stability of soluble hydrogenase at various temperatures The soluble enzyme preparation was held at the indicated temperatures for 15 min and then was cooled to 0° Assays were conducted at 37°

RESULTS AND DISCUSSION

Aside from the light activation of the hydrogenase of *P vulgaris*[1,2], the only other report of such activation is in the photosynthetic bacterium, *Rhodospirillum rubrum*, in which the light effect was shown to be due to an increased permeability of the cell to the substrate[13,14] To show that the light activation of *P vulgaris* is due to a direct effect on the hydrogenase and not to an increased permeability, the activation was demonstrated in cell-free systems. Table I shows that hydrogenase in a cell-free extract exhibits the same activation in light as whole cells The hydrogenase in this organism is particulate[3], the activity is sedimented at 100 000 × *g* and the particulate hydrogenase is activated by light (Table I) Since the particles are complex structures, one cannot be certain that the light directly affects the hydrogenase rather than alters the structure of the particle in some manner which increases the hydrogenase activity The hydrogenase was, therefore, solubilized and further purified

By treating it with deoxycholate and incubating it at pH 8 0, more than 80% of the recovered hydrogenase was solubilized This soluble hydrogenase showed the same light activation as whole cells and particles (see Table II) The stability of the soluble hydrogenase at various elevated temperatures is shown in Fig 2 Even at 75° for 15 min, more than 50% of the activity was recovered For purification, the enzyme preparation was heated at 60° for 10 min and then was precipitated with $(NH_4)_2SO_4$

TABLE I

LIGHT ACTIVATION OF CELL-FREE HYDROGENASE

The sonic extract was obtained by centrifugation at 13 000 × *g* of cells disrupted by sonic oscillation This extract was then centrifuged for 90 min at 100 000 × *g* Specific activity is expressed as units per mg protein The light activation is the ratio of the exchange activity in the light to the exchange activity in the dark

Fraction	*Specific activity*	*% of total activity*	*Light activitaon*
Whole cells	1 47	100	1 34
Sonic extract from 13 000 × *g*	2 22	91	1 39
Supernatant from 100 000 × *g*	0 38	7	—
Residue from 100 000 × *g*	1 99	51	1 38

After heating, the hydrogenase was precipitated at 0 4 saturation, in contrast, before heating, it precipitated at 0 6 saturation This step afforded a 3-fold purification, and the enzyme exhibited the same light activation The material was then chromatographed on Sephadex G-200 (Fig. 1) and showed the original light activation The purification procedure yielding a 65-fold purification and the light activation of each fraction is summarized in Table II The fact that light activation was observed in all active fractions during the purification suggests that light directly affects the hydrogenase activity

All fractions listed in Table II were subjected to polyacrylamide disc gel electrophoresis Except for the last fraction, they all showed two bands of hydrogenase activity which differed only slightly from each other in mobility and had R_F values near 0 5 ACKRELL *et al* [10] also observed two hydrogenase bands from *P vulgaris* The enzyme fraction after Sephadex chromatography only showed one very intense band of hydrogenase activity When the gels were stained for protein, the chromatographed fraction showed the loss of a number of fast moving protein bands which were present in the other fractions

To elucidate the mechanism of light activation, the properties of the enzyme active in the dark and the light-activated one were compared If the activation was due to an association or dissociation of subunits or to a large change in shape, changes in the apparent molecular weight would be expected after illumination There was no

TABLE II

PURIFICATION OF HYDROGENASE

The purification procedure is described in MATERIALS AND METHODS

Fraction	*Specific activity*	*% of total activity*	*Light activation*
1 Sonic extract	1 2	100	1 30
2 100 000 × *g* supernatant from 60% $(NH_4)_2SO_4$	6 6	33	1 40
3 100 000 × *g* supernatant after heating and 40% $(NH_4)_2SO_4$	18 5	18	1 40
4 Sephadex G-200 chromatography	80 0	9	1 39

difference in the molecular weight (estimated using sucrose density centrifugation) between the light-activated enzyme and the one active in the dark, both of which had molecular weights of about 115 000 This molecular weight is rather large when compared to the value of 10 000 obtained for the soluble hydrogenase of *Desulfovibrio desulfuricans*[15] and may suggest that the Proteus hydrogenase is not a truly soluble protein but rather is a large fragment of the original particle It is possible that the hydrogenase of *P vulgaris* is much larger than that of *D desulfuricans* since the hydrogenase of *Clostridium pasteurianum* has been reported to have a molecular weight of 56 000 (ref 16)

The mechanism of hydrogen cleavage in *P vulgaris* was shown[17,18] to be a heterolytic splitting of H_2, resulting in the formation of an enzyme hydride and proton When the exchange reaction was studied in 100% ^2H_2O, the rate of the appearance of ^2HH was five times that of the appearance of $^2H_2$ whether the reaction was catalyzed by the enzyme active in the dark or the light-activated one Therefore, the mechanism of action of hydrogenase is not affected by light

There was no difference in the K_m values, for hydrogen the light-activated enzyme had a K_m of $2\ 46 \cdot 10^{-4}$ M, and the one active in the dark had a K_m of $2\ 55\ 10^{-4}$ M The v_{max} was greater for the light-activated hydrogenase than for that active in the dark, and therefore the observed rate of exchange was greater in the light

Any explanation of the light activation of the hydrogenase of *P vulgaris* must account for the observation that the only difference between the two forms of the enzyme is in the value of the maximum velocity, the molecular weight, K_m, and mechanism of hydrogen activation are the same One possibility, consistent with these findings, is that a fraction of the hydrogenase is inactive in the dark and is converted to the active form by light Since the light-activated enzyme is the same as the enzyme normally active in the dark, the result of irradiation is to increase the quantity of active enzyme, increasing the maximum velocity but not changing the other properties A similar result would be obtained if a fraction of the hydrogenase in the dark were inhibited by the presence of an unknown inhibitor and if light in some manner destroyed the inhibitor thereby effectively increasing the concentration of active enzyme

A third possibility is that light induces a conformational change in the enzyme active in the dark, thus converting it to a more active form However in this case one might expect, in addition to an increased v_{max}, a change in K_m, which is not observed At present, the available evidence does not permit one to choose between these possibilities

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Science Foundation (NSF-GB-8003), the American Cancer Society (Institutional grant IN-41) and the U S Atomic Energy Commission (AT (30-1) 1803) The authors express their appreciation to Dr D Rittenberg for helpful and stimulating discussions

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