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PARTIAL PURIFICATION AND PROPERTIES OF ALCOHOL DEHYDROGENASE FROM THE UNICELLULAR GREEN ALGA *CHLAMYDOMONAS MOEWUSII*

ERNST J. M. GRONDAL, AUGUSTIN BETZ and KARLHEINZ KREUZBERG*

Botanisches Institut der Universität Bonn, Kirschallee 1, D-5300 Bonn 1, West Germany

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Key Word Index—*Chlamydomonas moewusii*; Chlorophyceae; algal fermentation; alcohol dehydrogenase; reaction mechanism.

Abstract—Alcohol dehydrogenase from the unicellular green alga *Chlamydomonas moewusii* was purified *ca* 142-fold. Optimum enzyme activity was at pH 6.8 for NADH oxidation and at pH 9.1 for NAD reduction. The MW of the native enzyme was determined by gel filtration to be *ca* 94 000, whereas SDS gel electrophoresis indicated the existence of two enzyme subunits with a MW of 42 000. ADH activity was specific for NAD. The oxidation rate of different alcohols decreased with higher chain length compared to ethanol. Free sulphydryl groups as well as a metal ion were necessary for enzyme activity as seen by the influence of iodoacetamide and 1,10-phenanthroline, respectively. The K_m values estimated for the basic substrates were NADH 1.1×10^{-4} M, acetaldehyde 1.25×10^{-3} M (both at pH 6.8) and NAD 1.25×10^{-4} M, ethanol 2.22×10^{-2} M at pH 8.9. Kinetic analysis indicated that a Theorell–Chance mechanism was involved. No isoenzymic forms were observed in autotrophic algal cells. The role of the enzyme in starch fermentation of *C. moewusii* is discussed.

INTRODUCTION

The alcohol dehydrogenases (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) in yeast and in liver have been extensively investigated and characterized [1]. The higher plant, e.g. pea [2], peanut [3], melon, tomato [4], rape [5], and wheat [6], ADHs have been shown to have similar properties to the yeast and liver enzymes. ADH has been detected and partially purified from *Euglena gracilis* [7] and from the cells of *Astasia longa* after growth on ethanol [8, 9]. In contrast to these results, some strains of *Chlamydomonas*, which grow well on acetate as a carbon-source, could not be cultured on ethanol [10, 11]. However, the same species are able to ferment starch under anaerobic conditions producing ethanol, e.g. *Chlamydomonas moewusii* [12], *C. reinhardii* and *Chlorogonium elongatum* [13]. Attempts to detect ADH activity in ethanol producing algae were unsuccessful for *Hydrodiction* [14] and marine algae [15].

The question arises, how the cells of *Chlamydomonas* and *Chlorogonium*, which can grow on acetate but not on ethanol as a sole carbon source under photoheterotrophic or heterotrophic conditions, can produce ethanol. The reason for this puzzling behaviour may be: (a) the need of different ADHs for ethanol production and consumption; (b) the different localization for the catabolic and anabolic processes accompanied with transport problems; or (c) the modulation of enyme kinetics by changes in metabolic conditions.

The aim of this work was to demonstrate the presence of ADH in ethanol producing *C. moewusii* and to study its kinetic behaviour after partial purification.

*To whom correspondence should be addressed.

Abbreviations: ADH, alcohol dehydrogenase; DTE, dithioerythritol.

RESULTS

Purification

Preliminary experiments indicated that ADH from *C. moewusii* irreversibly lost most of its activity using conventional purification steps, such as ammonium sulfate precipitation. The enzyme could be purified, however, by affinity chromatography on Cibacron Blue-Sepharose and gel filtration (Table 1). *Chlamydomonas* cells (30 g) were suspended in 50 ml chilled 0.02 M potassium phosphate buffer (pH 7.1) containing 1 mM DTE. After homogenization by one pass through a French pressure cell, the cold homogenate was immediately centrifuged for 20 min at 48 000 *g* and stored at −28° overnight. The turbidity, caused particularly by chlorophyll-containing particles, was removed by centrifugation of the thawed sample for 90 min at 48 000 *g*. The supernatant (16 mg protein/ml) was immediately applied to a Cibacron column, equilibrated with 0.02 M potassium phosphate buffer (pH 7.1) containing 1 mM DTE. The column was washed with the same buffer until the *A* at 280 nm reached zero. Thereafter, fractions of 6 ml were collected at a flow rate of 10 ml/hr, using a linear NAD-gradient (0–5.0 mM) (Fig. 1). Fractions with ADH activity were pooled, concentrated by ultrafiltration in an Amicon cell and applied to a Sephadex G-25 column equilibrated with 0.1 M potassium phosphate buffer (pH 7.1), containing 1 mM DTE. The enzyme solution (9 mg protein/ml) was applied to an Ultrogel AcA column (1.6 × 85 cm) equilibrated with the same buffer and gel filtration was performed with a flow rate of 7 ml/hr (Fig. 2) collecting 1.25 ml fractions. Those fractions containing ADH activity were concentrated to 2 mg protein/ml. This last step resulted in a stable enzyme preparation which was used for characterization of the enzyme. Electrophoresis of this ADH preparation in native polyacrylamide gels revealed

Table 1. Purification of *C. moewusii* ADH

Fraction	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Purification (-fold)	Recovery (%)
Crude extract	1002	10.70	0.01	1	100
Supernatant after freezing/thawing	842	10.66	0.012	1.2	99
Cibacron Blue	18	6.90	0.38	35.5	64
Ultrogel AcA 44	2	3.33	1.51	142.0	31

All enzymes assays were carried out at 25°, pH 8.9, with NAD and ethanol as substrates.

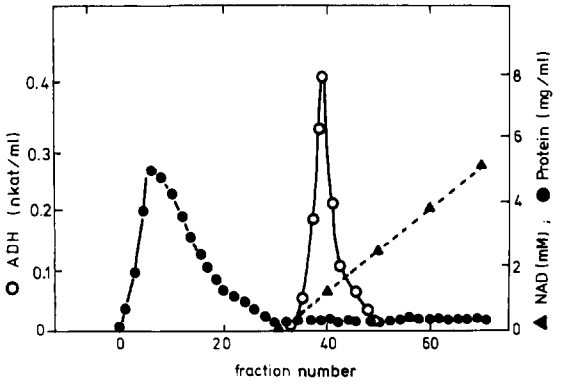

Fig. 1. Cibacron–Sepharose chromatography of *C. moewusii* ADH. The column was equilibrated with 0.02 M potassium phosphate buffer (pH 7.1) containing 1 mM DTE and eluted with a linear gradient of NAD in the same buffer as indicated. Fractions (6 ml) were collected and tested for enzyme activity. (●—●) Protein (mg/ml); (○—○) enzyme activity (nkat/ml); (▲---▲) NAD (mM).

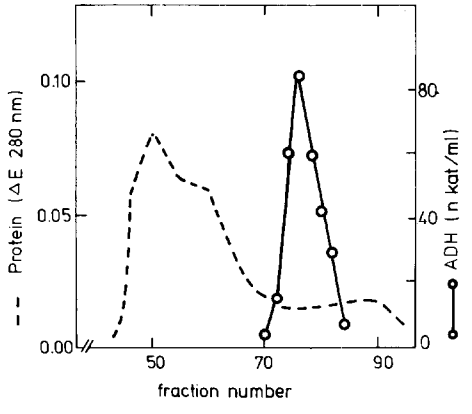

Fig. 2. Ultrogel AcA 44 chromatography of ADH. The column was equilibrated with 0.1 M potassium phosphate buffer (pH 7.1). Fractions were collected and tested for enzyme activity. (----) Extinction at 280 nm (measured continuously); (○) ADH activity (nkat/ml).

that it had several protein components after protein staining, whereas the ADH activity was confined to only one component. The enzyme became unstable if further purification steps were applied (e.g. ammonium sulfate precipitation, NAD-Sepharose chromatography).

Enzyme stability

Measurements of ADH activity in crude extracts of *C. moewusii* were difficult, when homogenization was carried out in buffer without sulphydryl reagents. DTE, 1 mM, stabilized the enzyme.

Influence of pH on ADH activity

The optimum pH for NADH oxidation was close to pH 6.8, whereas NAD reduction showed optimum activity at pH 9.1. Small changes in pH resulted in a rapid decrease of enzyme activity, especially in the range of pH 7–8. Maximum activity for NAD reduction reached only 48% of that for NADH oxidation, indicating that NADH oxidation was the favoured reaction under the conditions tested.

MW determination

The MW of the native ADH protein was determined by gel filteration on a calibrated Ultrogel AcA 44 column and was estimated as 94000. The MW of the subunits was determined by sodium dodecylsulfate electrophoresis which required further purification of the enzyme by gel electrophoresis of the native protein. The gel slices with ADH activity were eluted in SDS-sample buffer, boiled with 150 mM mercaptoethanol and gel electrophoresis in the presence of SDS was carried out. From the mobility of the enzyme a MW of 42000 was estimated. Therefore, *C. moewusii* ADH appears to be composed of two subunits with identical MWs of 42000.

In contrast to the enzyme from *A. longa* [8] and barley embryos [23] ADH from *C. moewusii* was highly specific for the coenzyme NAD, whereas NADP was inactive (Table 2). The oxidation rates of various alcohols were similar to those determined for the enzyme from rape [24]. While methanol and *iso*-propanol were oxidized at only 1% of the rate of ethanol, the rate of oxidation of other primary, unbranched alcohols decreased with increasing chain length.

The effect of sulphydryl group reagents

In order to determine whether the protection afforded by DTE was due to a requirement for sulphydryl groups, further experiments were performed with iodoacetamide. Enzyme activity was markedly inhibited in the presence of iodoacetamide. Inhibition was dependent on the concentration of iodoacetamide and was increased by preincubating the enzyme with the inhibitor before starting the reaction. Preincubation for 2 min under standard

Table 2. Substrate specificity of *C. moewusii* ADH

Substrate	Relative activity (%)
NAD	100
NADP	1
Methanol	1
Ethanol	100
Propan-1-ol	85
Propan-2-ol	1
Butan-1-ol	50
Propan-1,2-diol	58
Amyl alcohol	10

100% = 1 nkat/ml.

All alcohols were assayed at concentrations of 0.2 M; NAD, NADP at 2 mM; protein was 1.45 mg in the test vol.

assay conditions with 0.5 mM iodoacetamide resulted in a 50% inhibition, whereas the same rate of inhibition without preincubation required a concentration of 2.0 mM iodoacetamide. These data indicated the requirement of free sulphydryl-groups for catalytic activity of *C. moewusii* ADH. The inhibition by iodoacetamide could not be abolished by the simultaneous addition of NAD to the incubation mixture.

Effects of complexing agents

Enzyme activity of ADH from *C. moewusii* was readily inhibited by the chelating agents EDTA and 1,10-phenanthroline, as well as NaN_3. Concentrations of 5 mM EDTA and 0.2 mM NaN_3 gave a 50% inhibition of enzyme activity. These results indicate, that as in other ADHs [1] a divalent ion, such as zinc, may take part in catalysis. Inhibition caused by 1,10-phenanthroline was found to be non-competitive with respect to NADH and from the corresponding Dixon plot a K_i value of 1.1 mM was calculated. These results were in contrast to the data obtained for yeast and plant ADH [25–27], for which a competitive behaviour of 1,10-phenanthroline with respect to NAD and NADH was found.

Kinetic studies

Evidence for the type of kinetic mechanism in enzymatic bisubstrate reactions was drawn from the intersections in double reciprocal plots by the method of Lueck *et al.* [29] Thus, the intersection points for the plots of $1/V$ vs 1/[acetaldehyde] (Fig. 3a) and $1/V$ vs 1/[ethanol] (Fig. 3b) were on the abscissa. This indicated the existence of a random bi-bi or Theorell–Chance reaction mechanism, in *C. moewusii* ADH, as had been recently shown for the enzyme from rape [28]. The K_m values calculated from intercept replots of the data in the figures and were 1.25×10^{-4} M for NAD, 1.1×10^{-4} M for NADH, 2.2×10^{-2} M for ethanol and 1.09×10^{-3} M for acetaldehyde. These were comparable to those found for ADH from higher plants [6, 28]. By slope replots of the data in Fig. 3(a, b), as well as of the complementary primary plots with varied NAD concentration (data not shown), the dissociation constants for the binary complex of *C. moewusii* ADH were determined. The K_s for NAD

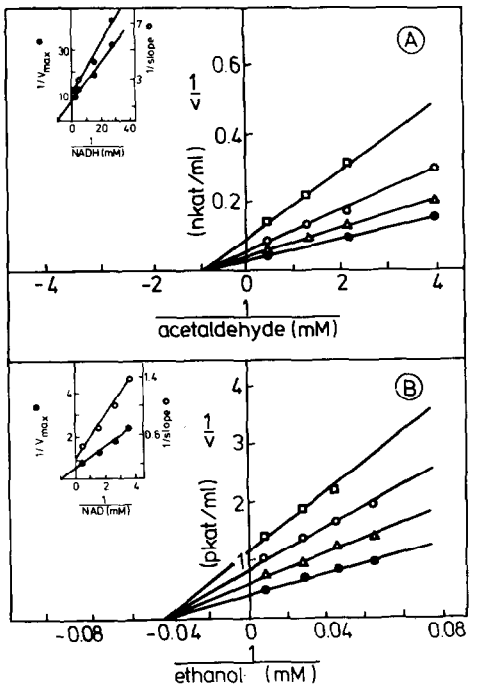

Fig. 3(A) Double reciprocal plot of the initial rate of NADH oxidation with varied acetaldehyde concentrations at fixed NADH concentrations (□) 0.036 mM; (○) 0.06 mM; (△) 0.18 mM and (●) 0.36 mM. Insert. Secondary plot of intercepts (●) and slopes (○) vs NADH concentration. (B) Double reciprocal plot of the initial rate of NAD reduction with variable ethanol concentrations at fixed NAD concentrations (□) 0.26 mM; (○) 0.36 mM; (△) 0.6 mM and (●) 1.8 mM. Insert. Secondary plot of intercepts (○) and slopes (●) vs NAD concentration.

was 2.73×10^{-4} M, for NADH and acetaldehyde, 8.9×10^{-3} M and for ethanol, 128×10^{-3} M.

The maximum NADH oxidation rate at pH 6.9 was 0.75 μmol/min·ml, whereas the maximum NAD reduction rate at pH 8.9 was 0.064 μmol/min·ml. However, the different reaction rates for both enzyme activities did not exclude the existence of a Theorell–Chance mechanism for *C. moewusii* ADH, since different conditions for catalytic directions were chosen. Binding of the first substrate to the enzyme can influence the affinity for the second substrate to the enzyme to some extent, as can be seen from the calculated interaction constants (K_m/K_s). In the forward direction, NADH lowered the affinity for acetaldehyde by 0.12, whereas acetaldehyde influenced the affinity for NADH by a factor of 0.012.

In the reverse direction, NAD lowered the affinity of the enzyme for ethanol only by 0.45 whereas ethanol influenced the enzyme interaction with NAD by a factor of 0.17.

DISCUSSION

The unicellular green alga *C. moewusii* contains an NAD-specific ADH with properties similar to the enzymes from yeast and higher plants. The enzyme is rather labile and sensitive to high salt concentrations, as is found for ADHs from rape [5], wheat [6], pea [23] and *A. longa*

[9]. Purification has to be carried out very rapidly in order to obtain a stable enzyme preparation. In contrast to the data reported for *A. longa* [8] and *E. gracilis* [7], ADH from *C. moewusii* is specific for NAD, while NADP is a poor substrate (Table 2). Its substrate specificity for various alcohols is similar to that shown by the enzymes from yeast and higher plants rather than those from other algae.

These studies provided no information on the presence of different ADH isozymes or the possible compartmentation of ADH activities in extracts of autotrophically grown *C. moewusii* cells. Comparison of ADH activities in cells grown under autotrophic and heterotrophic conditions as well as a more sophisticated separation of intact cell compartments are necessary to give definite answers to these questions.

The data obtained with 1,2-phenanthroline, NaN_3 and EDTA provided evidence that *C. moewusii* ADH is a metalloprotein (presumably containing zinc ions). Inhibition with 1,10-phenanthroline indicates a non-competitive pattern with regard to NADH. This is not in agreement with the results of Plane and Theorell [25], Dalziel [26] and Stiborova and Leblova [27] for ADH from yeast and pea, which gave a clearly competitive inhibition pattern. A possible explanation for our results might be the observation of Sund and Theorell [1] that the inhibitor causes a time dependent change in protein conformation and dissociation of subunits. Free sulphydryl groups are necessary for maintenance of catalytic activity of *C. moewusii* ADH as shown by results with iodoacetamide, whereas ADH from *E. gracilis* is not influenced by iodoacetamide [7].

The MWs reported for ADHs from plants differ considerably. While a MW of 60 000 for the plant enzyme is often found, higher values are obtained for the enzymes from *C. moewusii* (90 000), tea (95 000 [30]), wheat (116 000) [6] and *A. longa* (150 000 [9]).

In the unicellular green alga, *C. moewusii*, ethanol and acetate are produced simultaneously by fermentation of starch under anaerobic conditions in the dark [13]. The observed pH optimum at pH 6.8 for NADH oxidation fits well with the acidification of cell cytoplasm caused by acetate and the physiological function of ADH. On the other hand, the observed lack of ethanol uptake in the investigated species might be a consequence of the high pH optimum for ethanol oxidation (at pH 9.1) combined with a significant decrease of enzyme activity.

From the kinetic data it may be concluded that the constitutive ADH found in autotrophic cells is more important in the production of ethanol than in its utilization. The kinetic results suggest a Theorell–Chance mechanism similar to that found for rape ADH [27], rather than an ordered bi-bi mechanism as described for yeast ADH [1].

As our results clearly show, ethanol can be produced from acetaldehyde by a constitutive ADH in *C. moewusii* during the fermentation of starch. The reoxidation of glycolytically produced reduction equivalents seems to be the limiting factor for starch degradation under anaerobic conditions and in this way determines the reaction velocity of the complete Embden–Meyerhof pathway. Direct limitation of fermentation by the ADH concentration is unlikely in view of the described results, since enzyme activity in extracts of *C. moewusii* surpasses 40-fold the rate of ethanol production observed *in vivo* [13].

EXPERIMENTAL

C. moewusii strain 11-5/10 was obtained from the Pflanzenphysiologisches Institut der Universität Göttingen, West Germany. Growth conditions, harvesting and maintenance of the stock culture were as described elsewhere [16].

Cibacron–Sepharose chromatography. Coupling of Cibacron F3G-A to Sepharose 4B followed the method of Böhme *et al.* [17]. After loading the enzyme on the column (2 × 20 cm) elution proceeded with a linear gradient of 200 ml 0–5 mM NAD in 0.02 M KPi buffer (pH 7.1), containing 1 mM DTE. Fractions with ADH activity were collected and concd with a Diaflo PM 10 membrane in an Amicon cell. For removing NAD molecules the concd soln was applied to a Sephadex G-25 column (Pharmacia, PD-10) previously equilibrated with 0.1 M KPi buffer (pH 7.1), containing 1 mM DTE.

Ultrogel AcA 44 chromatography. Subsequent chromatography was performed on an Ultrogel AcA column (1.6 × 85 cm) equilibrated with 0.1 M KPi buffer (pH 7.1), containing 1 mM DTE; fractions containing ADH activity were concd by means of collodion bags (SM 132 00). MW determination was performed on the Ultrogel AcA 44 column after calibration with standard proteins of the following MWs: yeast ADH, 136 000; bovine serum albumin, 68 000; ovalbumin, 42 000 and chymotrypsinogen, 22 800. Void vol. was determined with Dextran blue. K_{av} value was calculated from the equation, $(V_e - V_0)/(V_t - V_0)$, where V_e = elution vol. and V_t = bed vol.

Polyacrylamide gel electrophoresis. PAGE of the native protein was carried out using the gels and buffer system described by Maurer [18]. After electrophoresis, the gels were stained for ADH in a soln containing 75 mM Hepes, 1 mM phenazine-methosulphate, 0.4 mM nitrobluetetrazolium, 0.72 M EtOH and 0.68 mM NAD.

For proteins the gel was stained with Coomassie blue as described by Chrambach *et al.* [19].

SDS-gel electrophoresis was carried out using the system of ref. [20]. Standard proteins used for MW determination were yeast phosphorylase (100 000), bovine serum albumin (68 000), catalase (58 000), ovalbumin (42 000), yeast ADH (34 000) and chymotrypsin (25 700). After staining for protein, the gels were scanned at 600 nm in a spectrophotometer (Gilford) and the R_f values calculated.

Enzyme assay. ADH activity (oxidizing) was measured spectrophotometrically by monitoring the increase of NADH *A* at 366 nm. The reaction mixture contained in 2.2 ml: 149.6 μmol $Na_4P_2O_7$–glycine buffer, pH 8.9; 4.4 μmol NAD; 0.22 nmol EtOH and usually 1.6 mg protein. The rate of NADH oxidation was determined using an assay mixture containing in 2.2 ml: 209 μmol KPi buffer, pH 6.9; 0.79 μmol NADH; 5.06 μmol acetaldehyde and 1.6 mg protein. The reaction mixture was incubated at 25° unless otherwise stated. For testing dependency of pH, enzyme activity was determined in 0.095 M KPi buffer (pH 5.0–9.0) and 0.068 M $Na_4P_2O_7$–glycine buffer (pH 8.5–10.0). Enzyme activity is expressed as nkat/mg protein. Deviations from this protocol are indicated in the text.

Protein determination. Protein concn was determined by means of the Biuret reagents following the method of ref. [21].

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