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METHYLAMINE DEHYDROGENASE OF *PSEUDOMONAS* sp. J

ISOLATION AND PROPERTIES OF THE SUBUNITS

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Summary

Two kinds of subunits, light subunit (M_r = 13 000) and heavy subunit (M_r = 40 000), were isolated from a methylamine dehydrogenase (M_r = 105 000) of *Pseudomonas* sp. J. The isolation of the subunits was carried out by gel chromatography after the enzyme had been treated with 3 M guanidine · HCl. Coexistence of both of the subunits was essential for the restoration of the enzyme activity. The heavy subunit exhibited an absorption maximum only at 278 nm but the light subunit had absorption maxima at 330 nm (shoulder) and 430 nm in addition to the peak at 278 nm. The results indicate that the prosthetic group, assumed to be a derivative of pyridoxal, was bound to the light subunit. The spectral changes of the light subunit were observed by addition of methylamine. Various physical and biochemical parameters of the subunits are reported.

Introduction

In a previous paper, we reported that methylamine dehydrogenase purified from *Pseudomonas* sp. J was found to consist of two different kinds of subunits by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [1].

This paper reports further experiments on the subunit structure of the enzyme. The isolation of the subunits from the native enzyme and some physicochemical properties of the subunits are described.

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Materials and Methods

Chemicals. All chemicals used in this study were reagent grade, and were obtained from commercial sources as reported in the previous paper [1]. Guanidine · HCl was purchased from Nakarai Chemicals, Ltd.

Methylamine dehydrogenase. The enzyme was prepared according to the previous paper [1] from *Pseudomonas* sp. J, which had been isolated from soil in our laboratory.

Methods. Enzyme assay, determination of protein concentration, SDS-polyacrylamide gel electrophoresis, determination of molecular weight, optical measurements, amino acid analysis and measurement of isoelectric point were those described in the previous paper [1].

Results

Reversibility of the enzyme activity. As a subunit structure of the enzyme was suggested from the previous work, the effect of reagents which dissociate proteins was examined (Fig. 1). The effective dissociating reagents were guanidine · HCl, KSCN, and KI. Those enzyme activities inactivated with these reagents were restored again to 5—85% of their original level by dilution of the denaturant concentrations with 1 M potassium phosphate buffer, pH 7.5 (Fig. 2). These results indicate that guanidine · HCl is the most suitable reagent for dissociating the enzyme without irreversible inactivation. The ability to

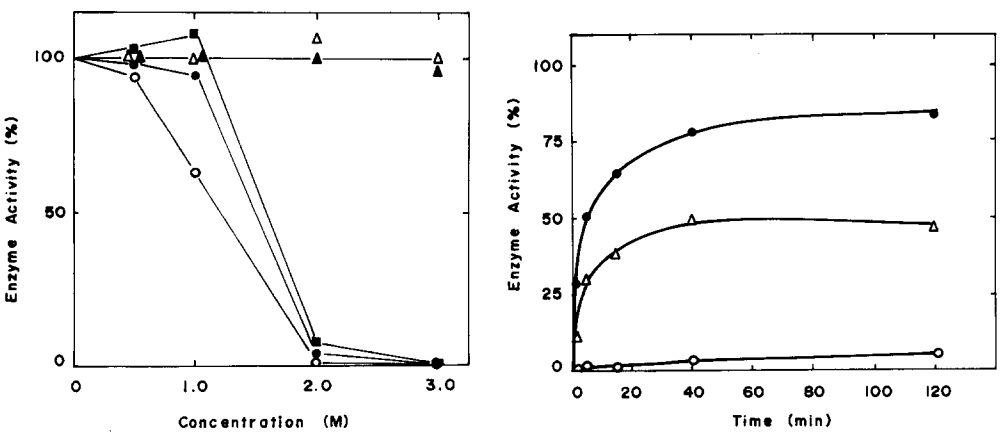


Fig. 1. Inactivation of the enzyme activity by various reagents. Each mixture (0.1 ml) contained the native enzyme (51 μg) and the reagent indicated in the figure. After standing at 30°C for 90 min, the sample mixture was measured for enzyme activity according to the standard assay conditions: ▲———▲, urea; △———△, KBr; ■———■, KI; ●———●, guanidine · HCl; and ○———○, KSCN. The concentrations of the reagents were diluted 200-fold in the assay mixture and their corrections were not made. The untreated enzyme activity was taken as 100%.

Fig. 2. Restoration of the enzyme activity. The enzyme (51 μg) was incubated with 3 M KI, 3 M guanidine · HCl and 3 M KSCN in 0.1 ml for 4.5 h at 30°C. The incubated solutions were diluted 10-fold with 1 M potassium phosphate buffer, pH 7.0, and 0.1 ml of the diluted solutions were assayed for the enzyme activity at the times indicated in the figure. ●———●, guanidine · HCl; △———△, KI; and ○———○, KSCN.

restore the enzyme activity was present even after incubation of the enzyme with 3 M guanidine · HCl for several days at 4°C.

Isolation of the subunits. 8 mg of the enzyme protein were treated with 3 M guanidine · HCl in 1 ml of 50 mM pótassium phosphate buffer, pH 7.5. for 2 h at room temperature. Then the solution was applied to a Sephadex G-100 column (2.5 × 42 cm) equilibrated previously with the same buffer containing 0.5 M guanidine · HCl, at 5°C (an elution profile is shown in Fig. 3). Two protein fractions were collected, those containing protein were pooled and dialyzed overnight at 5°C against 1 l of the buffer (changed three times). The dialyzed solutions were concentrated to a small volume in a collodion bag. (The protein with lower molecular weight will be referred to as the "light subunit" and that with higher molecular weight the "heavy subunit".) The isolated

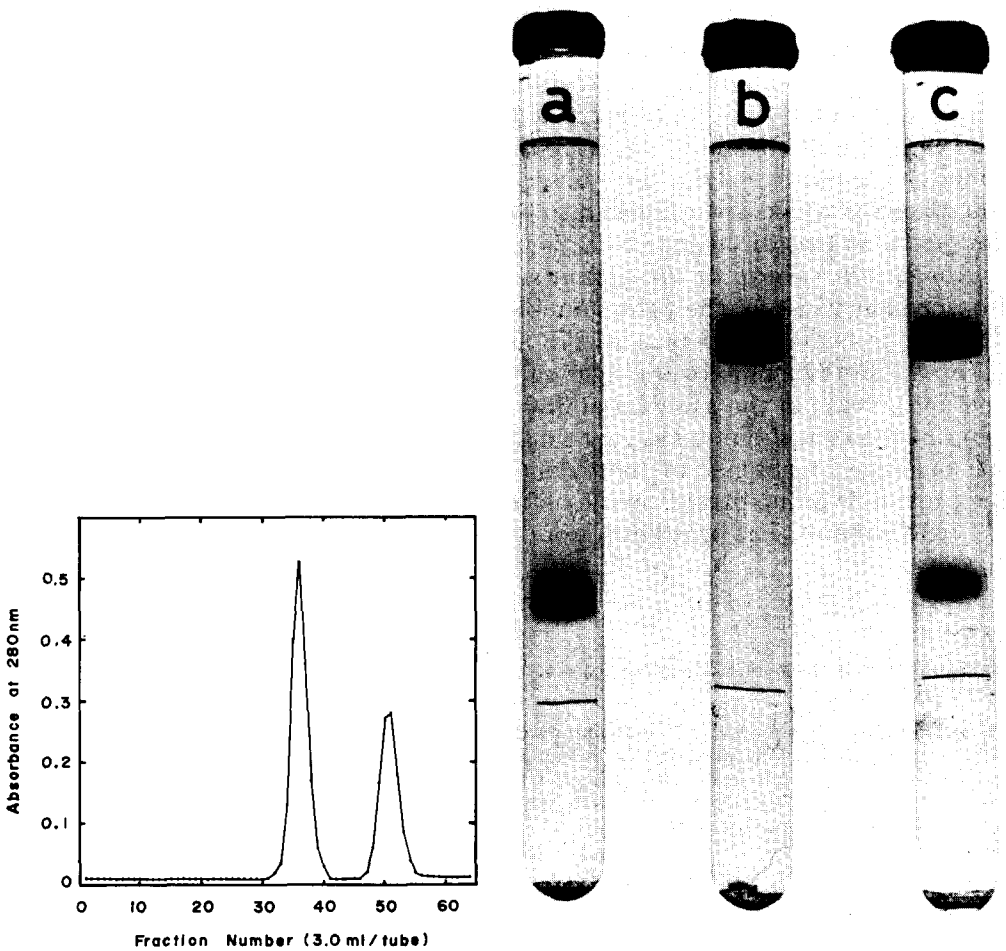


Fig. 3. Elution profile of the subunits by Sephadex G-100 column chromatography; see text.

Fig. 4. SDS-polyacrylamide gel electrophoresis of the subunits. The light subunit (60 μg), heavy subunit (40 μg), and native enzyme (40 μg) were treated under the same conditions as in Fig. 3. The left-hand gel (a) was stained for the light subunit, the center gel (b) for the heavy subunit, and the right-hand gel (c) for the native enzyme.

subunits were subjected to SDS-polyacrylamide gel electrophoresis to test homogeneity. The results (Fig. 4) reveal a single protein band for both subunits on the gels. The yields of both the subunits were about 95% for the light subunit and 83% for the heavy subunit of the native enzyme.

Molecular weight of the subunits. SDS gel electrophoresis and gel filtration on a Sephadex G-100 column suggested molecular weights of 13 000 and 40 000—50 000 for the light and heavy subunit, respectively.

Requirement of both subunits for enzyme activity. Neither the light subunit nor the heavy subunit alone had significant enzyme activity, if the separation of the two subunits was complete but recombining the subunits regenerated the activity (Table I). When the heavy subunit was added to a constant amount of the light subunit, a maximal enzyme activity was attained at a molar ratio for the two subunits near unity.

Isoelectric points and amino acid compositions. The isoelectric points of the light and heavy subunits were determined to be 5.1 and 8.5, respectively, by isoelectrofocusing. These values are lower than the 9.0 of the native enzyme. The amino acid compositions of the subunits are shown in Table II. More acidic amino acids than basic ones are found in the light subunit and the reverse is true in the heavy subunit. Most of the cysteines, determined as cysteic acid, found in the native enzyme were observed in the light subunit. The polarities of the subunit proteins are calculated to be 46% from Table II [3] and their values are similar to that of the native enzyme.

Absorption spectra of the subunits. The heavy subunit had a single absorption peak at 278 nm; the light subunit had a shoulder at 330 nm and a peak at 430 nm, in addition to a peak at 278 nm as shown in Fig. 5. By the addition of 0.75 mM methylamine to the light subunit, a decrease in the peak at 430 nm and an increase in the shoulder at 330 nm were observed. The absorption spectrum of the light subunit was very similar to that of the native enzyme in either the presence or absence of methylamine. These results strongly indicate that the prosthetic group present in the native enzyme is bound solely and tightly to the light subunit. The prosthetic group was not liberated from the light subunit

TABLE I

NECESSITIY OF THE PRESENCE OF BOTH THE HEAVY AND LIGHT SUBUNITS FOR THE ENZYME ACTIVITY

The light subunit, the heavy subunit, and mixtures of the two subunits were incubated for 60 min at 30°C in 0.35 ml of 0.8 M potassium phosphate buffer, pH 7.5. Then the enzyme activities were measured by the standard assay conditions except that the final concentration of the phosphate buffer was 280 mM instead of 100 mM. Amounts of the subunit protein are indicated in parentheses.

Subunits	Enzyme activity ($\Delta A_{600\ nm}$/min)
Light subunit (2.1 μg)	0
Heavy subunit (6.0 μg)	0
Light subunit (2.1 μg)	
plus heavy subunit (3.0 μg)	0.095
plus heavy subunit (6.0 μg)	0.292
plus heavy subunit (9.0 μg)	0.350
plus heavy subunit (12.0 μg)	0.375

TABLE II

AMINO ACID COMPOSITIONS OF THE SUBUNITS

The values are expressed as the nearest integers which are averages of the three determinations. Detail was the same described previously [1] except the value for tryptophane. Tryptophan was determined by the method of Simpson et al. [2].

Amino acid	Heavy subunit (residues/ 40 000 g)	Light subunit (residues/ 13 000 g)	$(\text{Light subunit})_2$ plus $(\text{Heavy subunit})_2$	Native enzyme *
Lys	32	5	74	69
His	9	2	22	21
Arg	15	5	40	40
Trp	4	1	10	9
Asx	37	17	108	105
Thr	19	10	58	56
Ser	22	13	70	70
Glx	37	8	90	87
Pro	19	9	56	62
Gly	27	13	80	77
Ala	28	7	70	68
Cys	2	10	24	27
Val	29	5	68	66
Met	9	1	20	21
Ile	12	5	34	34
Leu	37	6	86	80
Tyr	10	5	30	34
Phe	19	3	44	45

* Taken from previous report [1].

by heat treatment even in the presence of methylamine, as also shown with the native enzyme [1].

Fluorescence and circular dichroism spectra. When the heavy subunit was subjected to excitation at 287 nm, an emission maximum at 338 nm was

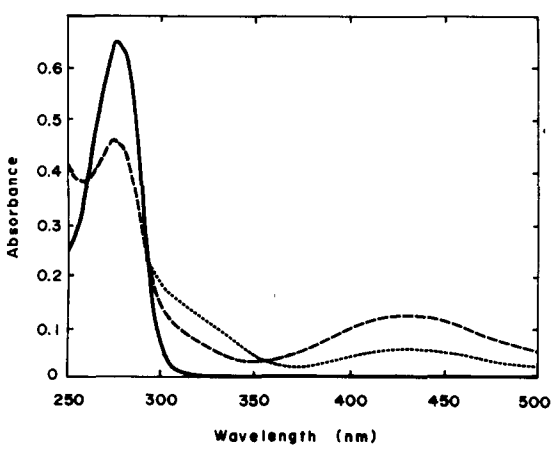


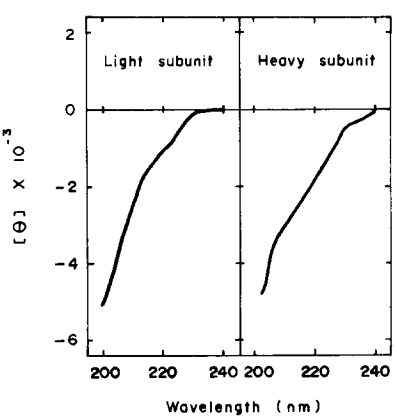
Fig. 5. Absorption spectra of the subunits. The spectra were measured in 50 mM sodium/potassium phosphate buffer, pH 7.0; ———, the heavy subunit (0.87 mg/ml) was contained in a cuvette having a 10 mm light path; - - - - - -, the light subunit (0.41 mg/ml) was contained in the cuvette; · · · · · ·, 0.75 mM methylamine hydrochloride was added to the light subunit.

observed, which may be due to the four tryptophan residues located in the hydrophilic center of the heavy subunit. For the light subunit, an excitation maximum at 318 nm and an emission maximum at 381 nm were obtained by similar methods. The emission maximum of the light subunit was not clearly observed when excited at 287 nm, although a tryptophan was present in the light subunit. In the presence of methylamine, both the excitation and emission maximum due to the prosthetic group in the light subunit were either intensified or stabilized, accompanied by red-shift of the maxima by 10 nm. The two emission maxima at 333 and 370 nm in the native enzyme [1] might be considered to be the sum of the maxima of the heavy and light subunits.

The circular dichroism spectra (200—250 nm) of both the subunit were very similar to each other (Fig. 6) and to that of the native enzyme [1]. This might be explained on the basis of the large content of random structure in the native enzyme and both subunits.

Thermal and pH stabilities. The results of thermal stability studies of the two subunits are shown in Fig. 7. The heavy subunit was more heat labile than the light subunit and its activity was almost completely lost at 50°C for 15 min at pH 7.1. On the other hand, the gradual inactivation of the light subunit began at 60°C, and even at 95°C, about 20% of the enzyme activity still remained.

No activity of the light subunit was lost at pH values from 3.0 to 9.0 but it was lost slightly at 10.6. The heavy subunit was unstable at acidic pH (below 4.0), but stable at pH 4.0—10.6. The stability of the native enzyme was greater



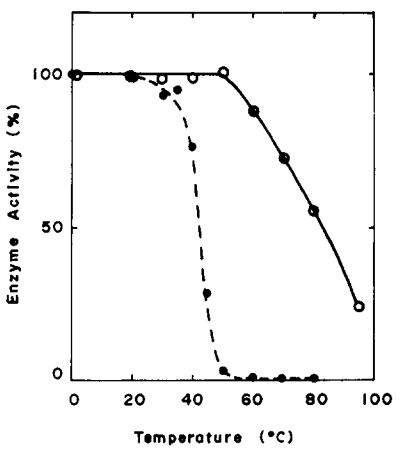


Fig. 6. Circular dichroism spectra of the light and heavy subunits. The subunits were dissolved in 50 mM potassium phosphate buffer, pH 7.0. Measurements were made in a cell of 0.63 mm light path length for the heavy subunit (0.37 mg/ml) and 1.03 mm light-path length for the light subunit (0.18 mg/ml) at 20°C.

Fig. 7. Thermal stabilities of the light and heavy subunits. The light subunit (9.0 μg) and heavy subunit (24.5 μg) were separately incubated in 0.15 ml of 67 mM potassium phosphate buffer, pH 7.1, for 15 min at the temperature indicated in the figure. For thermal stability of the light subunit, the heat-treated light subunit was incubated with the heat-untreated heavy subunit (24.5 μg) in 1.0 ml of 0.8 M potassium phosphate buffer, pH 7.5, for 1 h at 30°C; and then 0.2 ml of the incubation mixture was used for enzyme assay (———). For the heavy subunit, the heat-untreated light subunit (9.0 μg) was used and assayed as described above (- - - - -).

than that of the heavy subunit in both thermal and pH treatments [1], which suggests an inherent stability in the natural form of the enzyme.

Discussion

Methylamine dehydrogenase has been first purified from *Pseudomonas* AM1, a facultative methylotroph by Eady and Large [4,5]. Its properties were extensively studied but its subunit structure was not examined. Dahl et al. [6] reported that a methylamine dehydrogenase similar to that of *Pseudomonas* AM1 is present in methylamine-grown cells of *Pseudomonas* strain W1, which is an obligate methylotroph. However, the properties of this enzyme were not reported in detail.

We have purified a methylamine dehydrogenase from *Pseudomonas* sp. J, an obligate methylotroph, and have reported that the enzyme is very similar to that of *Pseudomonas* AM1 but differs in molecular weight and stability in thermal and pH treatments. Furthermore, we found that the enzyme is composed of two non-identical subunits [1].

The enzyme activity was lost in the presence of reagents, such as, guanidine · HCl, SCN^-, and I^-; and restored by dilution of these reagents in the presence of PO_4^{3-} and SO_4^{2-}.

The enzyme is composed of non-identical subunits and its structure is probably of the $\alpha_2\beta_2$ type. Questions which arise are: what are the roles of the light and heavy subunits in enzyme catalysis; and what is the physiological significance of the enzyme existing as a subunit enzyme in the cell? From the results of absorption spectra and the almost complete recovery of the enzyme activity from the denatured enzyme only by dilution, it is proposed that the prosthetic group of the enzyme was bound tightly and solely to the light subunit; and that by the addition of methylamine, the spectrum of the light subunit could be changed in the absence of the heavy subunit. These results indicate that the heavy subunit is not essential for the binding of methylamine to the light subunit, assuming the enzyme reaction proceeds according to the reaction mechanism proposed by Eady and Large [5] but is absolutely required for the subsequent reaction.

It is interesting to compare the light subunit from *Pseudomonas* sp. J with the isolated chromophore reported by Eady and Large [5]. They reported that the isolated chromophore exhibited a yellow color and migrated towards the anode on high-voltage paper electrophoresis at pH 9.5; also that it was dialyzable and gave no color reaction with ninhydrin. The light subunit in the present work was found to be an acidic, yellow and low molecular weight protein. These results indicate that the light subunit of methylamine dehydrogenase from *Pseudomonas* sp. J closely resembles the chromophore isolated from the enzyme *Pseudomonas* AM1 [5], except for ninhydrin reaction.

The conditions for reconstitution of the catalytically active enzyme from the isolated subunits and the properties of the reconstituted enzyme are now under investigation.

Acknowledgement

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