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Physiological Studies of Methane- and Methanol-Oxidizing Bacteria: Comparison of a Primary Alcohol Dehydrogenase from *Methylococcus capsulatus* (Texas Strain) and *Pseudomonas* Species M27

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A primary alcohol dehydrogenase has been purified from *Methylococcus capsulatus* (Texas strain). The purified enzyme catalyzes the oxidation of methanol and formaldehyde to formate; other primary alcohols are oxidized to their corresponding aldehydes. Ammonium ions are required for enzyme activity. The enzyme has a molecular weight of 120,000 daltons and consists of two 62,000 molecular-weight subunits which dissociate at acidic *p*H. The enzyme is similar to an alcohol dehydrogenase enzyme isolated from *Pseudomonas* sp. M27.

In a previous report, we described some of the physiological properties of the "Texas" strain of *Methylococcus capsulatus* with particular reference to the oxidation of C-1 compounds (11). Methane, methanol, formaldehyde, and formate were oxidized by cell suspensions to carbon dioxide; other primary alcohols were oxidized to their corresponding aldehydes. It was concluded that the oxidation of methanol, formaldehyde, and other primary alcohols is catalyzed by a nonspecific primary alcohol dehydrogenase (PAD) which is independent of pyridine nucleotides and requires ammonium ions for activation. Although the enzyme has been described for other methanol-oxidizing bacteria (12), the enzyme has only been purified from *Pseudomonas* M27 (4).

The present report describes the purification of PAD from *M. capsulatus*. Some comparisons of the biochemical and physical properties of the purified enzyme from *M. capsulatus* are made with the enzyme from a taxonomically unrelated microorganism, *Pseudomonas* M27.

MATERIALS AND METHODS

Organisms. *M. capsulatus* (Texas strain) was reisolated from old contaminated cultures and maintained in liquid medium under methane and air as described previously (11). *Pseudomonas* M27 was kindly provided by L. J. Zatman (University of Reading, England) and maintained on a mineral salts medium with methanol as sole carbon source (2).

Assay of enzyme activity. In some experiments PAD activity was assayed manometrically (3). Reaction mixtures (total volume of 3.0 ml) contained: 200 μmoles of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (Sigma Chemical Co.) buffer, *p*H 9.0; 50 μmoles of ammonium chloride; 2 μmoles of phenazine methosulfate (PMS); 10 μmoles of substrate and enzyme. In other experiments, PAD activity was determined by spectrophotometric assay (4). Reaction mixtures (total volume of 3.0 ml) contained: 150 μmoles of Tris-hydrochloride buffer, *p*H 9.0; 50 μmoles of ammonium chloride; 2 μmoles of PMS; 0.5 μmoles of 2,6-dichlorophenolindophenol (DCPIP); 20 μmoles of substrate and cell-free extract or purified enzyme. Formate was estimated with a formate dehydrogenase preparation from a "soluble" crude cell extract of *M. capsulatus* (11). Samples were incubated with the extract under conditions for the standard manometric assay in which formate is converted quantitatively to carbon dioxide (11). Aldehydes were estimated as the 2,4-dinitrophenylhydrazones (7) which were then recrystallized and characterized by melting-point determinations.

Enzyme units and specific activity. A unit of enzyme activity was defined as the amount of enzyme required to give an absorbancy change of 0.01 per min at 600 nm. The rates were determined 15 sec after the addition of substrate. Specific activities

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reported are expressed as nanomoles of DCPIP reduced per minute per milligram of protein. A molar extinction coefficient for DCPIP of 1.91×10^4 M^{-1} cm^{-1} was used (6). Protein concentrations were determined by the Folin-Ciocalteau method with bovine serum albumin as standard (10).

Purification of alcohol dehydrogenase. *M. capsulatus* and *Pseudomonas* M27 were grown at 37 and 30 C, respectively, in 14-liter carboys containing 10 liters of basal salt medium supplemented with methanol (0.5%, v/v). Cells in 50 mM potassium phosphate buffer, *p*H 7.0, were disintegrated, and crude cell extracts were obtained as described previously (11). The crude extract was then adjusted to *p*H 4.0 by the slow addition of 0.5 N HCl with continuous stirring at 2 C; a heavy precipitate which formed was removed by centrifugation at 90,000 × *g* for 30 min. The clear supernatant solution was then adjusted to *p*H 7.0 with 0.5 N NaOH. The enzyme was further purified by collecting the protein which precipitated on fractional addition of ammonium sulfate in the 45 to 80% saturation range. The protein was then dissolved in 20 mM Tris-hydrochloride buffer, *p*H 8.0, and passed through a column of diethylaminoethyl (DEAE) cellulose (25 by 2 cm) equilibrated with the same buffer. The protein which was eluted in the void volume was applied to a column of Sephadex G-200 (40 by 5 cm) equilibrated in 20 mM Tris buffer, *p*H 8.0 (see Fig. 1).

Ultracentrifugation. Sedimentation velocities of protein solutions were measured at 20 C in a 4°, 12-mm cell in the Spinco model E ultracentrifuge at 59,780 rev/min.

Immunoelectrophoresis. Immunoelectrophoresis of purified enzyme preparations in 50 mM barbital buffer, *p*H 8.6, was carried out by the method of Scheidegger (13). Antiserum to crude alcohol dehydrogenase from *M. capsulatus* was prepared in random-bred New Zealand rabbits. Two injections of 2 mg each of the crude enzyme incorporated in complete Freund's adjuvant (Difco) were given subcutaneously at 2-week intervals. One week later and at weekly intervals thereafter the rabbits were bled by marginal ear vein puncture and given booster injections of 1 mg of the antigen.

Acrylamide gel electrophoresis. Disc-gel electrophoresis was performed as described by Hay et al. (8). Untreated enzyme preparations were subjected to electrophoresis on 7% gels with 0.1 *M* Tris-glycine buffer, *p*H 8.9, for 2 hr at 4 ma/gel. The gels were stained with aniline blue black (1%, w/v, in 7% acetic acid) and destained in 7% acetic acid.

The polypeptide composition of the purified enzymes was determined by the procedure of Summers et al. (14). Sodium dodecyl sulfate (SDS) and urea were added to the enzyme preparations to yield a final concentration of 1% and 0.5 M, respectively. After incubation at 45 C for 1 to 2 hr, the samples were dialyzed for 48 to 72 hr against 8 liters of 10 mM sodium phosphate buffer, *p*H 7.1, containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol.

SDS-polyacrylamide gel (5, 7.5, 10%) electrophoresis was performed in glass tubes (0.6 by 12.5 cm) on verticle 10-cm separating columns and 0.5-ml (3.5%) stacking gels. Samples with bromophenol blue marker were subjected to 3 ma/gel for 7.5 to 8 hr. The gels were stained with 0.5% Coomassie blue and destained in 7% acetic acid- 40% methanol.

Molecular weight. The molecular weight of purified PAD from *M. capsulatus* and M27 was determined by Sephadex gel filtration (G-200) as described by Andrews (1). The elution volume (V_e) for various proteins of known molecular weights and the purified enzymes were determined and the K_{av} value for each was plotted as depicted in Fig. 6 and 8.

RESULTS

The purification procedure of PAD from *M. capsulatus* as described in this report is essentially the same as that used to purify PAD from M27 (4). A slightly wider range in ammonium sulfate concentration was used in the salt fractionation step. A final 14.3-fold purification of the enzyme was achieved, resulting in a yield of 55% (Table 1). Gel filtration on Sephadex G-200 gave a major protein fraction consisting of enzyme and a minor fraction showing absorption characteristics of a *c*-type cytochrome (Fig. 1). The PAD from M27 was purified as described by Anthony and Zatman (4); a 10-fold purification of this enzyme was achieved.

Several criteria were used to assess the purity and homogeneity of the enzyme preparations. By ultracentrifugal analyses, the schlieren profiles of the purified PAD revealed a single symmetrical peak with sedimentation coefficient ($S_{20,w}$) of 7.8 and 7.7 for the M27 and *M. capsulatus* enzymes, respectively. A single protein staining band was developed after electrophoresis on acrylamide gels at *p*H 8.9 (Fig. 2). Finally, rabbit antiserum prepared against the crude enzyme preparation of *M. capsulatus* revealed a single precipitin arc after immunoelectrophoresis of the purified enzyme.

The absorption spectrum of PAD from *M. capsulatus* with optima at 280 and 350 nm (Fig. 3) was similar to that reported previously for PAD from M27.

In preliminary studies, it was noted that the activity of PAD decreased rapidly when stored at 2 or −22 C (Fig. 4). The enzyme is, however, stable at these temperatures if stored in the presence of 10 mM methanol. Also, the presence of methanol facilitates the purification procedures.

Enzyme activity. As shown in Table 2, the PAD of *M. capsulatus* catalyzes the oxidation of primary alcohols to their corresponding aldehydes. However, it was also noted that formaldehyde, the expected product of methanol oxidation, is further oxidized to formate. These results suggested the unique possibility that

TABLE 1. *Purification of primary alcohol dehydrogenase from Methylococcus capsulatus (Texas strain)*

Enzyme fraction	Volume (ml)	Total protein (mg)	Total units	Specific activity[a]	Per cent yield	Purification
Crude extract	76	1,040	145,600	221	100	1.0
*p*H 4.0 Supernatant fluid	74	163	114,000	1,106	78	5.0
Ammonium sulfate (45–80% saturation)	25	140	100,000	1,185	69	5.4
DEAE cellulose	35	115	92,000	1,264	63	5.7
Gel filtration	60	40	80,000	3,160	55	14.3

[a] Values listed are nanomoles of 2,6-dichlorophenolindophenol reduced per minute per milligram of protein. Similar specific activities were obtained with methanol or formaldehyde as substrate. In the final fraction, the specific activity with formaldehyde was 2,370.

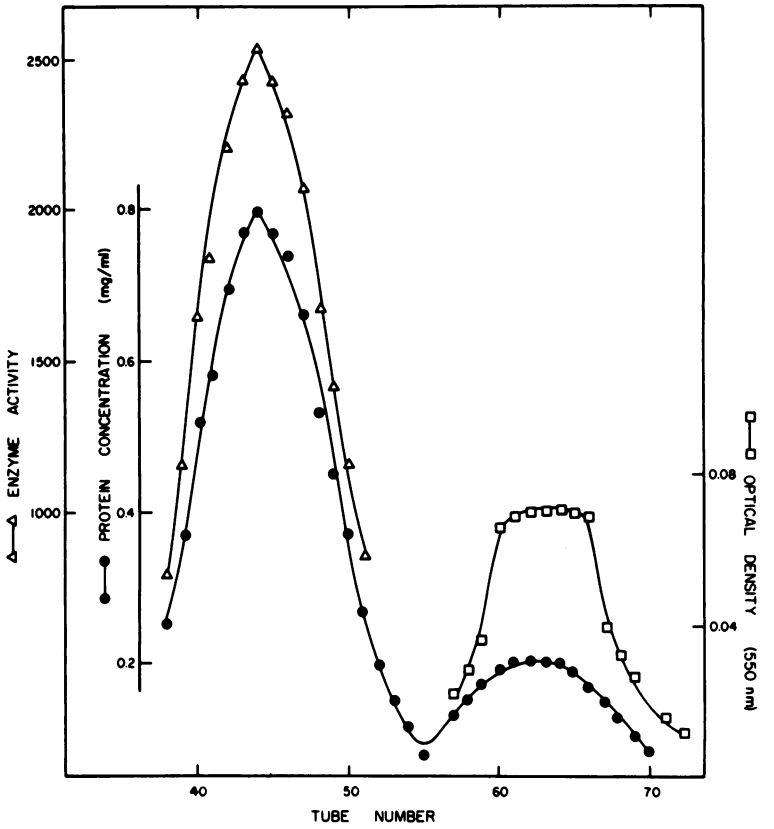

FIG. 1. *Gel filtration of active primary alcohol dehydrogenase fractions from DEAE cellulose chromatography. The protein was passed through a column (40 by 5 cm) of Sephadex G-200 equilibrated with 200 mM Tris-hydrochloride buffer, pH 8.0; 7.0-ml fractions were collected. Protein concentration (milligrams per milliliter) was estimated from the absorbancy at 260 nm and 280 nm. The enzyme activity of each fraction is given as the nanomoles of 2,6-dichlorophenolindophenol reduced per minute per milliliter of fraction. The absorbancy at 550 nm reflects the presence of c-type cytochromes.*

the same enzyme is operative in the catalytic oxidation of primary alcohols and formaldehyde. It was of interest therefore to compare the specific activities of PAD for methanol and formaldehyde at different *p*H values and at various ammonium ion concentrations (Tables 3 and 4). The ratio of specific activities for methanol and formaldehyde did not differ in the range of *p*H or ammonium ion concentration tested. The question of whether a common active site was involved in the oxidation of primary alcohols and formaldehyde was

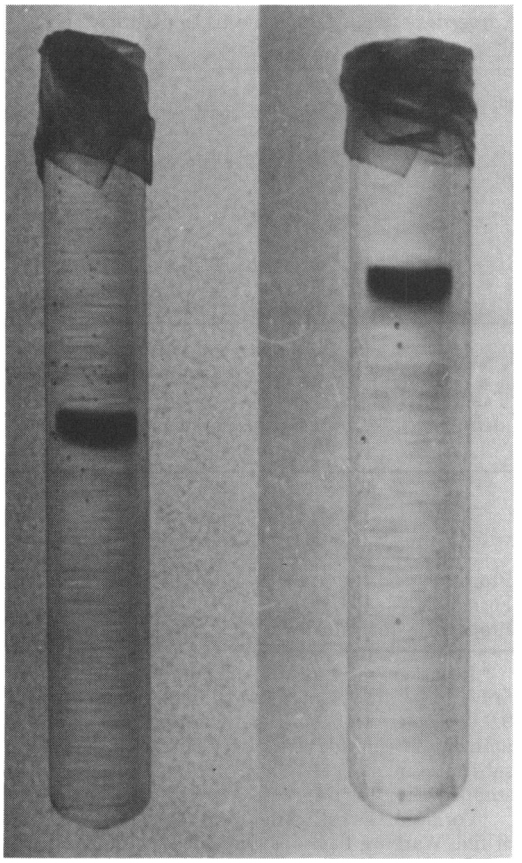

FIG. 2. *Disc-gel electrophoresis of purified primary alcohol dehydrogenase from M. capsulatus (left) and Pseudomonas M27 (right). Each enzyme preparation (75 μg) was subjected to electrophoresis for 2 hr at 4 ma/gel in 0.1 M Tris-glycine buffer, pH 8.9. The protein migrated from cathode (top) to anode.*

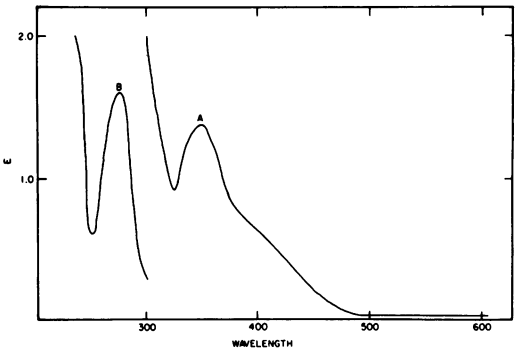

FIG. 3. *Absorption spectrum of the purified alcohol dehydrogenase of M. capsulatus was recorded with a Cary model 14 spectrophotometer. The protein was dissolved in 1.0 ml of 20 mM Tris-hydrochloride buffer, pH 8.0 at a concentration of 17 mg/ml (Tracing A) or 1.7 mg/ml (Tracing B). Ordinate scale is absorbancy per centimeter.*

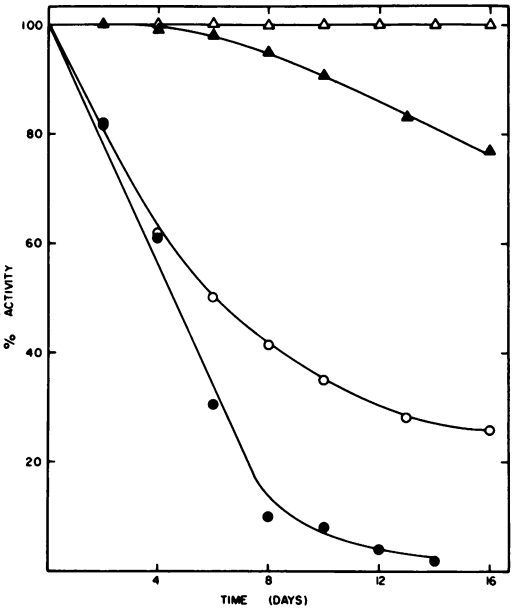

FIG. 4. *Stability of primary alcohol dehydrogenase from M. capsulatus. The activity of enzyme preparations was determined after storage for several days in the presence of 10 mmoles of methanol at 2 C (▲) and −22 C (△), or in the absence of methanol at 2 C (●) and −22 C (○).*

examined further by substrate competition experiments. Since methanol and formaldehyde are both oxidized to formate, it was necessary to utilize another primary alcohol, namely propanol, together with formaldehyde as competing substrates. The experiments consisted of determining the effect of propanol on the oxidation of formaldehyde to formate. As shown in Table 5, there was an 11-fold reduction in the amount of formate recovered. Since propionaldehyde was also produced, it was necessary to control this experiment further. Although not shown in the data, propionaldehyde was not an effective inhibitor of formaldehyde oxidation by PAD. In addition, neither propanol nor propionaldehyde was an effective inhibitor of the formate dehydrogenase used to assay formate production. Hence it appears that under the conditions of the experiment described here, propanol inhibited the conversion of formaldehyde to formate, presumably by successfully competing for the available reactive sites.

Molecular weight subunit structure. The molecular weight of purified PAD from *M. capsulatus* and M27 was determined by gel fil-

TABLE 2. *Substrate specificity of primary alcohol dehydrogenase from Methylococcus capsulatus (Texas strain)*[a]

Substrate	Product recovered	Per cent recovery	Specific activity	K_m (μM)
Methanol	Formate	85	3,160	0.1
Primary alcohol C2-C5	Corresponding aldehyde	67	2,645	0.6
Iso-butanol	0			
Sec-butanol	0			
Formaldehyde	Formate	80	2,370	0.6
Acetaldehyde	0			

[a] A 100-μmole amount of substrate was used for each assay.

TABLE 3. *Effect of pH on primary alcohol dehydrogenase activity of Methylococcus capsulatus (Texas strain)*

pH	Specific activity[a]		Ratio[b]
	Methanol	Formaldehyde	
6.0	0	0	
7.0	0	0	
7.5	245	185	1.32
8.0	750	565	1.30
8.5	1,835	1,375	1.33
9.0	3,160	2,370	1.30
10.0	2,250	1,680	1.34

[a] A 20-μmole amount of methanol or formaldehyde was used as substrate at each pH.

[b] Values listed are the ratios of specific activity of primary alcohol dehydrogenase for methanol/formaldehyde.

TABLE 4. *Ammonium chloride and methylamine activation of primary alcohol dehydrogenase from Methylococcus capsulatus (Texas strain)*

Activator[a]	Specific activity[b]		Ratio[c]
	Methanol	Formaldehyde	
Ammonium chloride			
0	0	0	
10	1,710	1,580	1.08
20	2,370	1,935	1.22
30	2,390	2,370	1.30
Methylamine			
0	0	0	
10	340	275	1.20
20	510	381	1.34
30	710	540	1.30
40	790	600	1.31

[a] Values listed are micromoles of ammonium chloride or methylamine per reaction volume of 3.0 ml.

[b] See footnote *a*, Table 3.

[c] See footnote *b*, Table 3.

TABLE 5. *Substrate competition for primary alcohol dehydrogenase of Methylococcus capsulatus (Texas strain)*

Substrate	Micromoles[a]	Formate recovered[b] (μmoles)
Propanol	500	0
Formaldehyde	75	13.2
Propanol + formaldehyde	500 + 75	1.2

[a] Reaction mixture for manometric assay contained: 100 μmoles of Tris-hydrochloride buffer, pH 9.0; 2 μmoles of phenozinemethosulfate; 50 μmoles of ammonium chloride; 0.5 mg of purified enzyme and substrate in a total 3.0-ml volume. Reaction was stopped after 30 min.

[b] Formate was estimated from the pooled products of five Warburg flasks as described in the Materials and Methods.

tration on a standardized column of G-200 (Fig. 5). From the linear portion of the plot, a molecular weight of 120,000 was estimated for each enzyme.

The subunit composition of the enzyme preparations was determined from disc-gel electrophoresis analysis of the reduced enzyme in the presence of 0.5 M urea, 0.1% SDS, and 0.1% mercaptoethanol. For each enzyme, a single protein band was identified by staining the gels with Coomassie blue. In a separate study, a mixture of the two enzymes was reduced and subjected to electrophoresis on 10 and 5% gels; a single band was developed. This indicated that the subunits for each enzyme were similar in molecular weight. The molecular weight of the subunits was also determined by comparing the migration of the reduced enzymes with the migration of other proteins of known molecular weight. As shown in Fig. 6, the molecular weight of the subunit was calculated as 62,000. Hence, it appears that PAD from *M. capsulatus* and from M27 consists of two subunits of identical molecular weight.

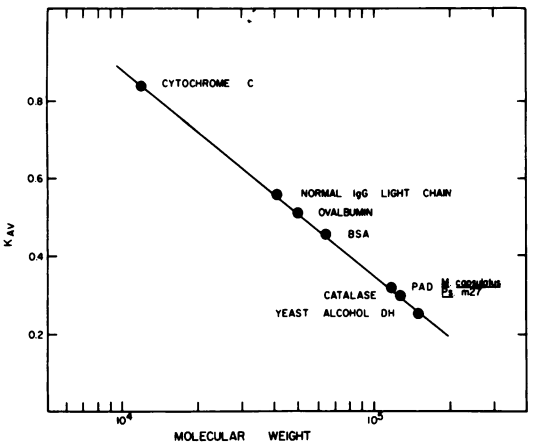

FIG. 5. *Gel filtration of known proteins and of primary alcohol dehydrogenase (PAD) from M. capsulatus and Pseudomonas M27. Column was equilibrated with 20 mM Tris-hydrochloride buffer, pH 8.0. The proteins used for calibration and the PAD from M. capsulatus and M27 were applied in separate determinations as described in the Pharmacia manual.* $K_{av} = (V_e - V_0)/(V_t - V_0)$, *where* V_e *is elution volume,* V_0 *is void volume determined with blue dextran 2000, and* V_t *is bed volume.*

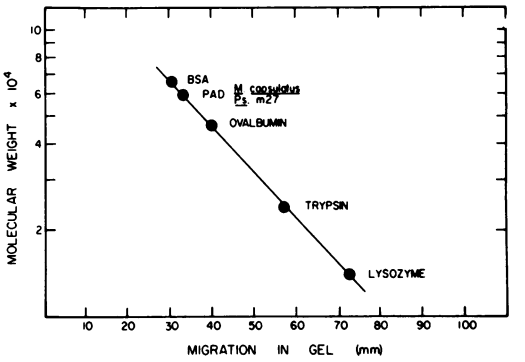

FIG. 6. *Disc-gel electrophoresis on 10-cm separating columns of 10% polyacrylamide gel containing SDS (0.1%), mercaptoethanol (0.1%), and urea (0.5 M). The proteins used for calibration and the primary alcohol dehydrogenase from M. capsulatus and M27 were subjected to electrophoresis at 3 ma/gel for 8 hr.*

Many active macromolecular proteins which are composed of subunits held together by noncovalent interactions can be dissociated by denaturing agents such as urea, SDS, or guanidine hydrochloride. In some cases, dissociation can be achieved by varying the pH or ionic strength of the solvent system. In the present study, the molecular integrity of PAD from *M. capsulatus* and M27 at acidic pH was assessed by ultracentrifugation and gel filtration. The effect of low pH was seen as a reduction in the $S_{20,w}$ values of the enzymes from 7.2 to 2.7 (Fig. 7); the sedimentation coefficient of the enzymes at pH 3.5 or 3.3 was not significantly different from that observed at pH 6.0. The schlieren profiles of the enzymes at the pH values examined showed only a single sedimenting component. Hence, complete dissociation of the enzyme occurs within a narrow pH range of 3.3 to 3.0. Gel filtration at low pH provided additional evidence that the enzymes dissociate to monomeric subunits at pH 3.0. A molecular weight of 62,000 was calculated for the monomers from a plot of the molecular weight and K_{av} for other proteins at pH 3.0 (Fig. 8). These results show that PAD from *M. capsulatus* and M27 is composed of two sub-

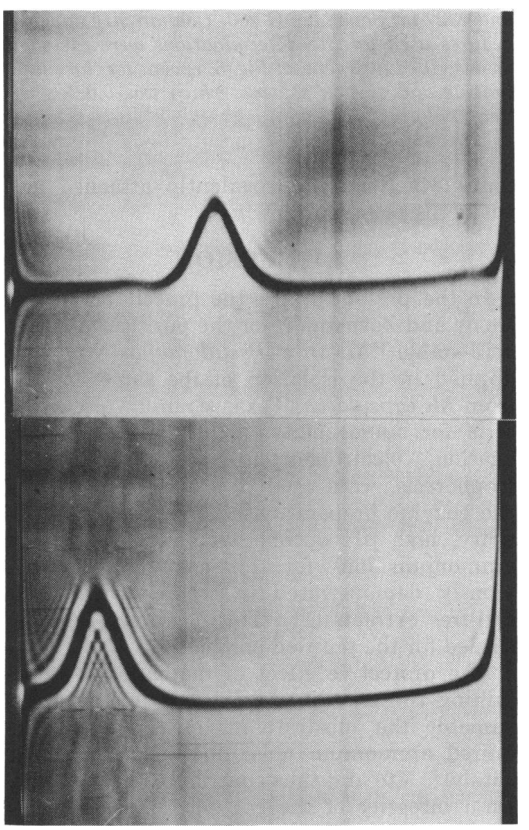

FIG. 7. *Schlieren patterns of the ultracentrifugation of primary alcohol dehydrogenase (PAD) from M. capsulatus. The sedimentation is from left to right at 59,780 rev/min. Photographs were taken after 48 min. Samples were run in 0.025 M sodium chloride, pH 6.0 (upper) and pH 3.0 (lower). The* $S_{20,w}$ *values for the upper and lower patterns are 7.2 and 2.7, respectively. Similar patterns were obtained with PAD from M27.*

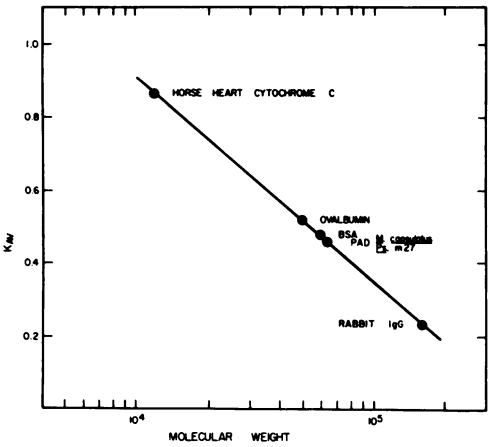

FIG. 8. *Gel filtration of calibration proteins and primary alcohol dehydrogenase from M. capsulatus and M27 on Sephadex G-200. Column size and procedures used for the determinations were the same as described in legend of Fig. 5, except for the equilibrating and elution solvent, which was 0.025 M sodium chloride, pH 3.0.*

units which are noncovalently attached and easily dissociated at low *p*H.

DISCUSSION

In the present study, the procedures of Anthony and Zatman (4) for the purification of an acid-stable PAD from *Pseudomonas* M27 were applied in the isolation of the same enzyme from *M. capsulatus* (Texas strain). Various criteria for homogeneity, including ultracentrifugation, electrophoresis, and immunoelectrophoresis, were used to assess the purity of the enzyme preparations. The substrate specificity, high *p*H optima, and requirement for ammonium ions for this enzyme were previously demonstrated in studies with crude cell-free extracts (11). This has now been confirmed for the purified enzyme.

The protective effect of methanol in maintaining the stability of PAD is unclear. Presumably the substrate in the absence of required ammonium ions binds at or near the catalytic site and thus contributes to the structural integrity of the enzyme. It would be of interest to determine whether other alcohols (including secondary and tertiary alcohols) are equally effective in this way.

The spectral properties of PAD from *M. capsulatus* and from M27 are similar in showing absorption maxima at 260 and 350 nm. Also, a pteridine derivative, unlike other pteridine cofactors, was detected in the *M. capsulatus* purified enzyme preparation (Urushibara, Forrest, Hoare, and Patel, *unpublished data*). Whatever the role of pteridine in methanol oxidation (5), it appears that the same mechanism is operative in both organisms.

The most interesting observation provided by studies with PAD from these two organisms is the ability of this enzyme to catalyze the oxidation of formaldehyde to formic acid. Since no other mechanism for catalyzing formaldehyde oxidation could be demonstrated in *M. capsulatus*, it is tempting to postulate that methanol (or other primary alcohols) and formaldehyde are oxidized by the same enzyme. This is consistent with the observation that the ratio of specific activities of PAD for methanol and formaldehyde is unchanged at various *p*H and ammonium ion concentrations and that the conversion of formaldehyde to formate could be inhibited by propanol. These results, however, do not exclude the possibility that a separate mechanism(s) for methanol and for formaldehyde oxidation may be occurring in the artificial assay system. Nevertheless, the dual and sequential oxidation of methanol to formic acid appears to be unique for the acid-stable alcohol dehydrogenase of M27 and *M. capsulatus*. In a separate study, Heptinstall and Quayle (9) also demonstrated that crude extracts of methanol-grown *Pseudomonas* AM-1 oxidized methanol and formaldehyde when phenazine methosulfate was used as an electron acceptor. Linkage of the dual activities was further suggested by studies with a methanol dehydrogenase-deficient mutant (*Pseudomonas* M-15A) which also was devoid of formaldehyde-oxidizing activity.

The enzymes from *M. capsulatus* and M27 are also similar in molecular weight (i.e., 120,000). In addition, each enzyme consists of two 60,000 molecular-weight subunits which are noncovalently attached to each other. Although the subunits are easily dissociated at *p*H 3.0, reassociation of the subunits by restoring the *p*H to 6.0 was unsuccessful as the enzyme tended toward aggregation and denaturation. This may be due to the release of the pteridine cofactor in the dissociation step as observed by Anthony and Zatman (5).

The enzymes differ, however, in their electrophoretic mobilities on disc-gel electrophoresis (Fig. 2). This suggests that the enzymes also differ in their acidic and basic amino acid compositions. In a separate study, a comparison of the mole per cent of the amino acid composition from the *M. capsulatus* and the mole per cent of amino acid composition previously reported by Anthony and Zatman (4)

from the M27 enzyme showed striking similarities except for charged amino acids. The differences noted are sufficient to account for the faster mobility of *M. capsulatus* enzyme on disc-gel electrophoresis. Another study will elaborate upon the serological similarities and differences between the two enzymes.

ACKNOWLEDGMENTS

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