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Purification and Characterization of Chemotactic Methylesterase from *Bacillus subtilis*†

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ABSTRACT: By utilization of methanol evolution as an assay, a protein methylesterase from *Bacillus subtilis* has been purified. A 1200-fold purification has been achieved by CM-Bio-Gel A, hydroxylapatite, and Bio-Gel P-60 column chromatography. Gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicate the enzyme is a monomer of 41 000 in molecular weight. The enzyme is stabilized and activated by aqueous glycerol solutions. Methyl-accepting chemotaxis proteins (MCPs) serve as substrates for the enzyme. The enzyme requires divalent cation for activity, with maximum activity obtained at 1.1 mM Mg^{2+}. The enzyme is most active at pH 7.5 and at 28 °C. Methylesterase has an apparent K_m for methylated MCPs of about 10 nM.

Posttranslational modification of polypeptides allows proteins to be rapidly modified without the expense of new protein synthesis. In the case where the modifications are reversible, transient changes can occur, and later, the unmodified protein can be regenerated. One such modification, methyl esterification, has been shown to play an important role in bacterial and leukocyte chemotaxis (Goy et al., 1977; Goldman et al., 1982; O'Dea et al., 1978) and is associated with secretion from the adrenal medulla (Gagnon et al., 1978b), posterior pituitary (Gagnon et al., 1978a), and parotid gland (Gagnon et al., 1979).

In the case of bacterial chemotaxis, the response to an increase in the concentration of an amino acid attractant is a change in degree of methylation of certain intrinsic membrane proteins, the methyl-accepting chemotaxis proteins (MCPs) (Goy et al., 1977; Goldman et al., 1982) on certain glutamate residues (Kleene et al., 1977; Burgess-Casler, 1982). During the period when the degree of methylation of MCPs is changing, the bacteria swim smoothly and, afterward, return to their normal erratic swimming. In the Gram-negative *Escherichia coli*, the MCPs become more methylated (Goy et al., 1977); in the Gram-positive *Bacillus subtilis*, the MCPs become less methylated, with production of methanol (Goldman et al., 1982).

Our laboratory has been involved in understanding the enzymology of the process of methylation and demethylation of the MCPs. Previously, we purified and characterized two methyltransferases that catalyzed transfer of methyl groups from *S*-adenosylmethionine to MCPs. In this paper, we describe the purification and characterization of a methylesterase that removes these methyl groups, with production of methanol (Goldman et al., 1982; Toews & Adler, 1979). Stock & Koshland (1978) first reported on the existence of a methylesterase involved in bacterial chemotaxis. Although the closely related enzyme from *E. coli* was found to be 38 000 in molecular weight (Silverman & Simon, 1977), G-100 chromatography on an extract from *Salmonella typhimurium* showed the enzyme to be 100 000 in molecular weight in its native form (Stock & Koshland, 1978). Pseudorevertant studies indicated that the methylesterase interacts with the *cheZ* gene product in *E. coli* (Parkinson, 1977). Thus, the 100 000 molecular weight activity observed on a gel-filtration column may represent either a homotypic or heterotypic multimer. It has also been observed that the methylesterase has the ability to carry out a second modification on proteins distinct from demethylation, possibly deamidation (Kehry & Dahlquist, 1982). Purification of the enzyme would allow a

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careful characterization of the enzyme and the reaction it catalyzes. We report here the purification of a methylesterase from *B. subtilis* that demethylates MCPs and results in methanol production.

Materials and Methods

Chemicals. *S*-Adenosyl[*methyl*-^{3}H]methionine (15 Ci/mmol) was purchased from Amersham Corp. or ICN. DEAE-Bio-Gel A, CM-Bio-Gel A, Bio-Gel HTP (hydroxylapatite), Bio-Gel P-100, and Bio-Gel P-60 were obtained from Bio-Rad Laboratories.

Bacterial Strains. OI 1085 is a chemotactically wild-type strain of *Bacillus subtilis*, and OI 1100 is a chemotaxis mutant derived from OI 1085 by mutagenesis (Ullah & Ordal, 1981b).

Media. Tryptone broth is 1% tryptone and 0.5% NaCl. L broth is 1% tryptone, 0.5% NaCl, and 0.5% yeast extract. Buffer used during cell disruption (FP buffer) is 20% (v/v) glycerol, 10 mM potassium phosphate (pH 7), 10 mM $MgCl_2$, 20 mM KCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.02% NaN_3, and 2 mM phenylmethanesulfonyl fluoride (PMSF). Buffer used in the in vitro assays (ME buffer) is 20% (v/v) glycerol, 2 mM potassium phosphate (pH 7), 0.2 mM $MgCl_2$, 0.02 mM EDTA, 0.2 mM 2-mercaptoethanol, and 0.02% NaN_3. Alternatively, MT buffer [as described previously by Ullah & Ordal (1981a)] supplemented with 20% (v/v) glycerol and 0.02% NaN_3 was also used in in vitro assays. Gel filtration was performed in the presence of MT buffer supplemented with 20% (v/v) glycerol and 0.02% NaN_3. Hydroxylapatite column buffer (HA buffer) consisted of 10 mM potassium phosphate, 1 mM EDTA, 1 mM NaN_3, 4 mM 2-mercaptoethanol, and 20% (v/v) glycerol.

Enzyme Purification. All steps of the purification were performed between 0 and 4 °C and carried out as rapidly as possible. Growth of bacteria was as described previously (Burgess-Cassler et al., 1982). Generally, 25–50 g (wet weight) of cells was used for the purification. The cells were washed 2 times with 1 M KCl (Nakayama et al., 1977) and once with FP buffer and resuspended in 150 mL of FP buffer. Cells were broken open by passing them through an Aminco French pressure cell 3 times. A pressure of 19 000 psi was utilized. Unbroken cells and debris were removed by centrifuging the above extract at 17600*g* for 20 min in a Beckman JA-20 rotor. The supernatant was immediately passed over a hemoglobin–Sepharose 2B affinity column to remove proteases (Nakayama et al., 1977). RNase and DNase at 15 and 20 μg/mL, respectively, were added to the column eluate, and membranes were removed by centrifugation at 120000*g* for 6 h in a Beckman 70 Ti rotor. The supernatant was dialyzed against ME buffer supplemented with 0.1 mM PMSF until a conductivity reading of less than 0.5 mmho was obtained.

The dialyzed cytoplasm was then passed over a CM-Bio-Gel A column equilibriated with ME buffer. This was followed by 100 mL of ME buffer. Bound material was eluted with a 400-mL linear gradient of 0–0.3 M KCl in ME buffer. Fractions of 5.5 mL were collected on a Gilson fraction collector. Fractions containing methylesterase activity were pooled and applied to a hydroxylapatite column equilibrated with ME buffer. Following the sample, 50–100 mL of HA buffer was run over the column. Material sticking to the column was eluted with a 400-mL linear gradient of 10–300 mM potassium phosphate in HA buffer. Those fractions containing peak methylesterase activity were pooled and concentrated to approximately 2.5 mL with an Amicon ultrafiltration cell equipped with a 10 000 molecular weight cut-off membrane. This material was then passed over a Bio-Gel P-60 column. Fractions with peak methylesterase activity from this column represented the purified enzyme.

Protein was monitored throughout the purification by measurement of the absorbance at 280 nm with a Hitachi Model 100-40 spectrophotometer. Protein concentration was determined as described previously (Ullah & Ordal, 1981b).

Samples were analyzed on sodium dodecyl sulfate (SDS)–polyacrylamide gels [10% acrylamide and either 0.125% bis(acrylamide), for visualizing demethylated product, or 0.375% bis(acrylamide), for visualizing proteins during purification] (Laemmli, 1970). Fluorography was performed by the method of Laskey & Mills (1975).

Substrate Preparation. Two different procedures were utilized to prepare radioactive methylated membranes. The first utilized OI 1085 membranes isolated as described under Enzyme Purification. Membranes were methylated by taking advantage of the methyltransferase enzyme sticking to them. Membranes (44 mg) were mixed with 4 mL of MT buffer, 30 μL of 1 M $MgCl_2$, and 50 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine. The reaction was allowed to proceed for 4 h at room temperature. The reaction was terminated by dilution with MT buffer supplemented with 0.5 M KCl, and the membranes were isolated by centrifugation. These membranes were washed with MT buffer, resuspended in 2 mL of MT buffer, and stored frozen (−70 °C) in small aliquots.

The second method for preparing membranes again used OI 1085 cells. The cells were broken open as described under Enzyme Purification, but the membranes were not separated from the cytoplasm. To 2 mL of this extract was added 25 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine. The reaction was allowed to proceed for 3 h at room temperature. The reaction was terminated, and membranes were isolated as described for the first procedure.

Enzyme Assay. Methylesterase activity was assayed by measuring methanol production. Conway diffusion cells were used to determine the amount of radioactive methanol produced (Goldman et al., 1982). The outer well of the Conway cell contained in a 60-μL volume 10 μL of radioactive membranes (0.22 mg) and various amounts of enzyme or buffer. Opposite this reaction mix was placed 50 μL of 2×-concentrated Laemmli sample buffer (Laemmli, 1970) and a glass bead. The center well contained 0.5 mL of water. The Conway cells were closed, Vaseline being used to obtain a tight seal. The reaction was allowed to proceed for 4 h at room temperature. The reaction was then terminated by rotating the Conway cells so the Laemmli sample buffer mixed with the reaction mix. The cells were allowed to sit overnight, they were opened, and 0.250 or 0.400 mL from the center well was analyzed for radioactivity by liquid scintillation spectroscopy. Visualization of the product was done by removing 50 μL of the stopped reaction and running this on SDS–polyacrylamide gels, which were subsequently fluorographed.

Results

Purification of the Methylesterase. Two assays were used to follow the purification of the methylesterase from *B. subtilis*. Methanol evolution was utilized to rapidly determine where methylesterase activity was in the various purification steps. Visualization of the demethylated proteins was accomplished by running the samples on SDS–polyacrylamide gels followed by fluorography. This allowed us to follow the enzymes purification and to determine if a change in substrate specificity might be occurring during the removal of extraneous proteins.

Table I presents the various steps used in purifying the methylesterase. The enzyme represents about 0.09% of the cytoplasmic proteins. In order to determine what conditions are required for good enzymatic activity, various buffer com-

Table I: Purification Scheme

step	total protein (mg)	total act. (pmol of CH_3OH produced/min)	sp act. (pmol of CH_3OH produced min^{-1} mg^{-1})	x-fold purification	yield (%)
(1) dialyzed cytoplasm	658	10.72	0.016	1	100
(2) CM-Bio-Gel A column	1.16	4.42	3.81	238	41
(3) Bio-Gel HTP column	0.303	2.02	6.67	417	19
(4) Bio-Gel P-60 column	0.0116	0.23	19.8	1237	2

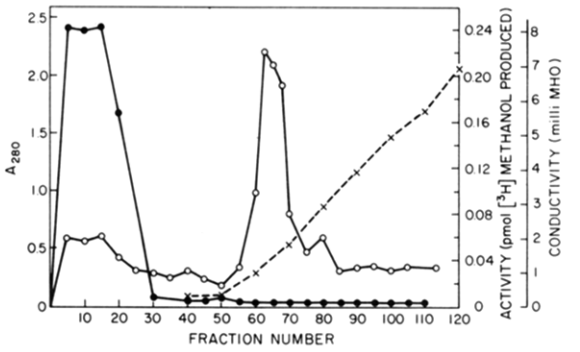

FIGURE 1: CM-Bio-Gel A chromatography of dialyzed cytoplasm. Plotted are A_{280} (●), activity (○), and conductivity (×) vs. fraction number.

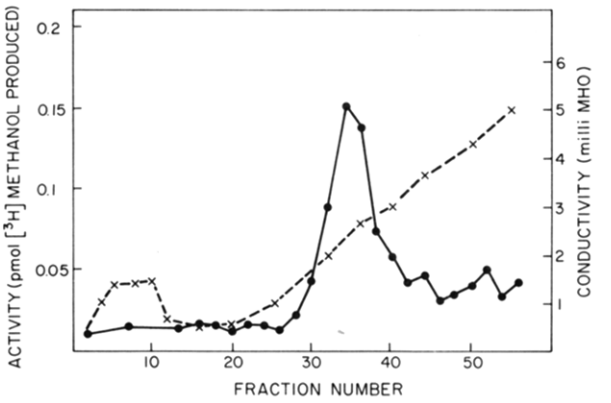

FIGURE 2: Hydroxylapatite chromatography of pooled (peak activity) CM-Bio-Gel A fractions. Plotted are activity (●) and conductivity (×) vs. fraction number.

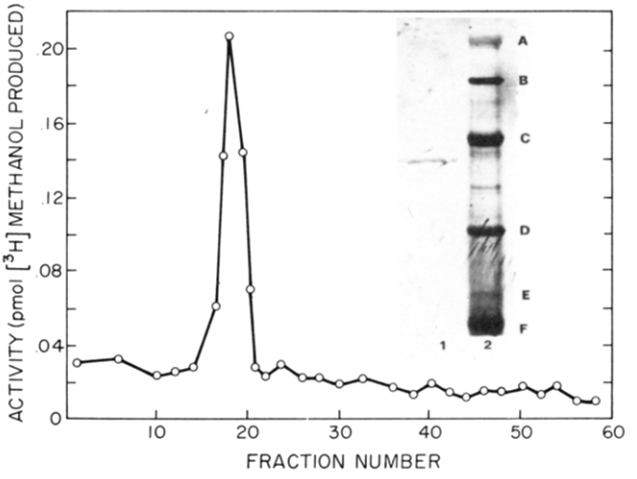

FIGURE 3: Bio-Gel P-60 chromatography of pooled (peak activity) hydroxylapatite fractions. Plotted are activity (○) vs. fraction number. (Inset) SDS–polyacrylamide gel electrophoresis of purified methylesterase. Proteins were visualized by silver staining (Merrel et al., 1981). (Lane 1) Fraction 18 from Bio-Gel P-60 column. (Lane 2) Molecular weight standards: (A) phosphorylase B, 92 500; (B) bovine serum albumin, 66 200; (C) ovalbumin, 45 000; (D) carbonic anhydrase 31 000; (E) soybean trypsin inhibitor, 21 500; (F) lysozyme, 14 400.

ponents were investigated. Presence of glycerol was found to result in more enzymatic activity and therefore was included in all buffers during the purification. The enzyme is a basic protein and stuck weakly to CM-Bio-Gel A (Figure 1), necessitating the use of a low ionic strength buffer (see Materials and Methods). We found that as one applies more protein to the column, the yield decreases. We attempted to stick more enzyme to the column by equilibrating the cytoplasmic extract and the column with a pH 5.5 acetate buffer. Although more protein was bound to the column, the enzyme activity was irreversibly diminished (see pH profile, Figure 7). Most of the cytoplasmic proteins are acidic and did not bind to the CM-Bio-Gel A column so that a 238-fold purification resulted.

Peak fractions from the CM-Bio-Gel A column were pooled and applied directly to a hydroxylapatite column (Figure 2). We have obtained good enzymatic activity from this column only when the elution buffer is HA buffer. Elution with ME buffer severely decreased enzymatic activity. The reason for this is not known. No detectable absorbance at 280 nm was observed (data not shown), indicating very little protein present at this stage of the purification. The increase in conductivity of the first few fractions is due to KCl in the sample, which was taken directly from the CM-Bio-Gel A column. Peak fractions from the hydroxylapatite column were pooled and concentrated to approximately 2.5 mL in an Amicon ultrafiltration cell.

The concentrated sample was applied to a Bio-Gel P-60 column (Figure 3). Methylesterase activity from the gel-filtration column corresponded to a protein with a molecular weight of 44 000. The purity of the enzyme from the gel-filtration column was determined by SDS–polyacrylamide gel electrophoresis (inset of Figure 3). The denatured enzyme ran as a single band with a molecular weight of 41 000. The purified enzyme was stored frozen at −70 °C in small aliquots. It retained good activity for at least 1 month.

Product Visualization and Time Course. The products of the methylesterase reaction are demethylated MCPs and methanol. Enzyme assays were carried out as described under Materials and Methods. In these assays, membranes were prepared as described for the second method under Substrate Preparation. This procedure results in all membrane-bound MCPs being methylated whereas the first method only results in one of the MCPs being methylated. Aliquots from the center well of a Conway diffusion cell were counted to determine methanol production, and aliquots from the outer well were run on SDS–polyacrylamide gels to visualize the product. We observed that the use of formaldehyde instead of 2× Laemmli solubilizer to stop the Conway cell reactions killed the enzyme, even before mixing. It appears the enzyme is extremely sensitive to formaldehyde, to the extent that the formaldehyde vapors inactivate it.

When pure enzyme or cytoplasmic extract was mixed with radiolabeled membranes, the label was released as methanol over the next few hours (Figure 4). Time-course experiments with the pure enzyme compared to cytoplasm indicate a similar

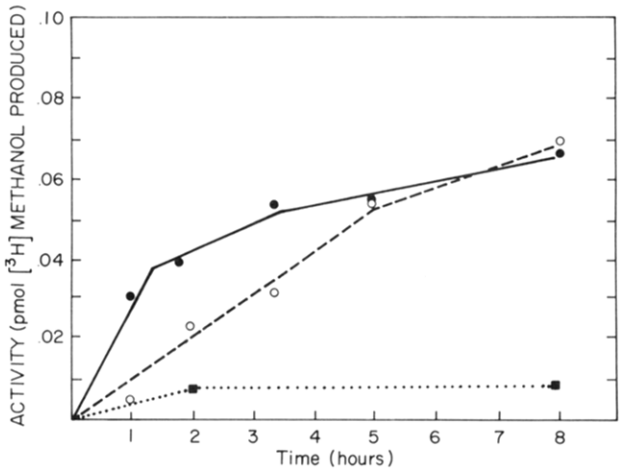

FIGURE 4: Time course of methanol evolution. Plotted are pure enzyme (0.03 μg of protein, corresponding to 0.59 fmol of CH_3OH produced/min) (●), cytoplasm (17 μg of protein, corresponding to 0.28 fmol of CH_3OH produced/min) (○), and buffer (■) activity vs. time.

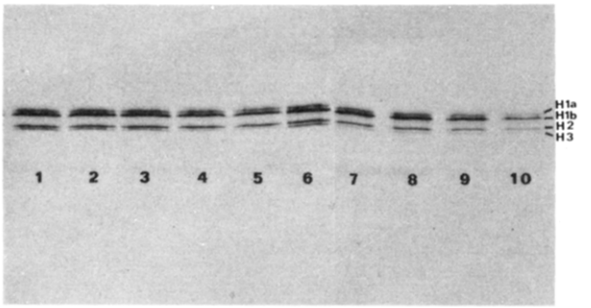

FIGURE 5: Time course of MCP demethylation. Cytoplasm (30 μg of protein): (1) 13 min; (2) 0.5 h; (3) 1 h; (4) 2 h; (5) 3 h. No enzyme: (6) 4 h. Pure enzyme (0.03 μg of protein): (7) 1 h; (8) 2 h; (9) 3 h; (10) 5 h.

substrate specificity (Figures 5 and 6), the smallest MCP (H3) being the most easily demethylated. Glutamic acid methyl ester and fluorescein mono-*p*-guanidinobenzoate are not hydrolyzed.

Enzyme Location. The methylesterase was found to be most prevalent in the cytoplasm. But, the enzyme activity was detected on isolated membranes and could be dissociated from the membranes upon 0.5 M KCl MT buffer washing (data not shown).

Temperature Optimum. Methylesterase activity was found to be very temperature sensitive. Maximum activity was observed at 28 °C, with half-maximal activity occurring at 20 and 37 °C. Standard assays were done at room temperature (22 °C) or about 60% of maximum activity.

Effect of pH on Methylesterase. Methylesterase was inactive at pH 5 and only slightly active at pH 6 (Figure 7). Activity greatly increased with pH until maximum activity was obtained at pH 7.5. Activity gradually decreased above pH 7.5.

Divalent Cation Requirement. Divalent cation was required for methylesterase activity, with the optimum being 1.1 mM. No activity was detected at 0.1 mM, and half-maximal values occurred at 0.4 and 3.2 mM. Thus, the requirement for Mg^{2+} seems cooperative, in view of the steepness of the rising part of the curve. In separate experiments, Mg^{2+} was found to promote binding of methylesterase to membranes (data not shown). Ca^{2+} was as effective as Mg^{2+} when substituted into the assay mixture (data not shown) (see paragraph at end of paper regarding supplementary material).

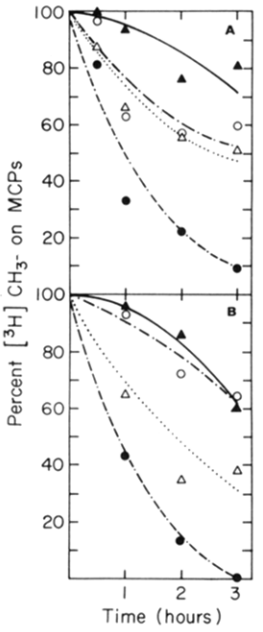

FIGURE 6: Densitometric depiction of bands H1a (▲), H1b (△), H2 (○), and H3 (●) of Figure 5. (A) Cytoplasm (lanes 2–5 of Figure 6). (B) Pure enzyme (lanes 7–9 of Figure 6).

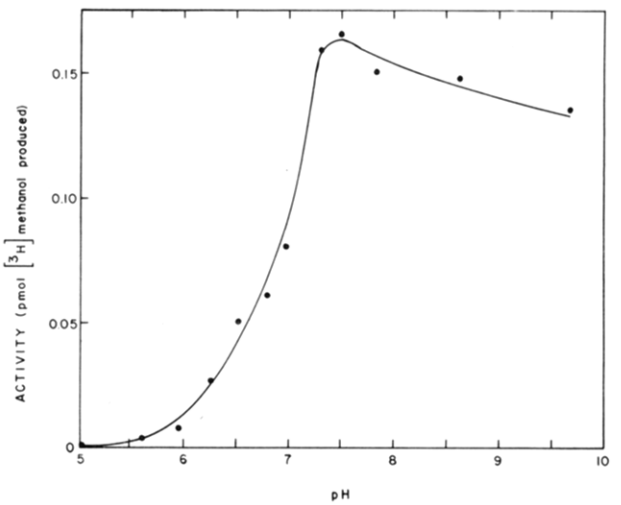

FIGURE 7: Effect of pH on methylesterase activity. Experimental conditions were as described except that a 200-μL reaction volume was employed and the pH was decreased by addition of 0.25 N HCl and increased by addition of 0.25 N KOH.

Apparent K_m of Methylesterase for Methylated MCP. Fully unmethylated membranes were isolated from OI 1100, a mutant lacking methyltransferase (Burgess-Cassler et al., 1982). These membranes were methylated in vitro with methyltransferase II and *S*-adenosyl[*methyl*-^{3}H]methionine (SAM) to generate methylated MCPs (Burgess-Cassler et al., 1982). These membranes were washed, resuspended in MT buffer, and frozen as described under Materials and Methods. The specific radioactivity (Ci/mmol of [^{3}H]methyl) of the methylated MCPs was the same as that of the methyl donor (SAM), since the [^{3}H]methyl esters are undiluted by preexisting esters.

Unmethylated mutant membranes did not inhibit methylesterase activity in the Conway cell assay (data not shown). Therefore, those MCPs remaining unlabeled in the prepared substrate did not inhibit the methylesterase enzyme.

Identical amounts of methylesterase were mixed with various amounts of this substrate in conway cell assays. A Lineweaver–Burk plot of $1/V_0$ vs. $1/[s]$ gave a straight line with

a correlation coefficient of 0.99 and indicated a K_m of 10 nM for methylated MCPs.

Discussion

We have purified a methylesterase from *B. subtilis*. The enzyme demethylates all membrane-bound MCPs that are methylated by the methyltransferase enzyme. The purification procedure resulted in a single enzyme activity being detected on a Bio-Gel P-60 column. Occasionally, we have noticed a second enzymatic activity eluting from the Bio-Gel P-60 column corresponding to a protein of 21 000 in molecular weight. We are determining the relationship of this protein to the 44 000 molecular weight enzyme in order to determine if it is a proteolytic product.

The methylesterase, like the methyltransferase (Burgess-Cassler et al., 1982) from *B. subtilis*, is a basic protein. Both enzymes show little affinity for a weak anion exchanger but much stronger affinity for a weak cation exchanger. Both enzymes also can be removed from membranes by 0.5 M KCl and MT buffer washing. The ease with which the enzymes are dissociated from the membranes with salt solutions implies an interaction that is mainly ionic. The fact that the MCPs are acidic proteins (Engström & Hazelbauer, 1980) and the enzymes are basic proteins is consistent with an ionic interaction.

Enzymatic activity was increased significantly by glycerol. Glycerol in solution retards protein unfolding due to water-mediated exclusion of glycerol from contact with the protein (Gekko & Timasheff, 1981). The net result is a tightly folded protein and an increase in protein–protein interaction (Gekko & Timasheff, 1981). Due to the stabilizing effect of glycerol on the methylesterase activity, it was included in all steps of the purification.

Divalent cation (Mg^{2+}) was found to be required of methylesterase activity. Furthermore, Mg^{2+} enhanced the binding of methylesterase to membranes in vitro. A likely mode of action for these ions is the formation of salt bridges to stabilize the association of methylesterase with membranes. KCl inhibits methylesterase activity possibly by interfering with the mode of action of Mg^{2+}.

Methylesterase has a pH optimum of 7.5, with activity sharply decreasing below this pH. This may be the result of protonation of a nucleophile necessary for activity. The enzyme has good activity over a narrow range of temperature, with maximum activity at 28 °C. Methylesterase has a K_m for methylated MCPs of about 10 nM. Since additional unmethylated membranes do not inhibit the enzyme, this K_m is not the result of the enzyme binding to membrane and then two-dimensionally searching for substrates. Methylesterase has a high affinity for methylated MCPs. Such affinity is not unusual for protein–protein interactions.

Bacteria respond to attractants by swimming for a certain length of time and then they return to prestimulus tumbling and swimming frequency (adaptation) (Berg & Tedesco, 1974). The length of time it takes bacteria to adapt has been correlated to methylation of MCPs in *E. coli* (Goy et al., 1977) and demethylation of MCPs in *B. subtilis* (Goldman et al., 1982). Thus, the adaptation enzyme in *B. subtilis* appears to be the methylesterase, unlike in *E. coli* where the methyltransferase serves this role. Using the in vitro system described in this paper to assay the methylesterase, we can now investigate the affect of attractants and other cellular components on this demethylation reaction.

It is interesting to note that the methylesterase from *E. coli* interacts with the *cheZ* gene product (Parkinson, 1977). There might exist two domains in the enzyme, one that has enzymatic activity and another that can interact with other proteins that may influence the enzymatic activity. Interaction studies with the *B. subtilis* equivalent of the *cheZ* gene product and the purified methylesterase may shed some light on this.

Reversible methylation reactions have been found in a number of eukaryotic as well as prokaryotic systems (Goy et al., 1977; Goldman et al., 1982; Gagnon, 1979; Quick et al., 1981). Recently, methylesterase activity has been detected in a number of tissues (Gagnon, 1979). Chemoattractants are found to stimulate demethylation of preformed methyl esters of proteins involved in rabbit neutrophil chemotaxis (Venkatasubramanian et al., 1980). Nonhistone chromosomal proteins are found to be methylated in a tissue-specific manner (Quick et al., 1981). Methylesterase activity has also been observed to be associated with chromatin (Quick et al., 1981). Thus, the involvement of reversible protein methylation in regulating various biological processes appears to be wide spread. The purification of the enzymes involved in these reactions and a characterization of their substrates will help in understanding the role of methylation in regulatory mechanisms. So far as we know, this work represents the first purification of a methylesterase from either prokaryotic or eukaryotic sources.

Supplementary Material Available

Three figures showing effects of temperature and magnesium ion concentration on methylesterase activity and a Lineweaver–Burk plot of methylesterase activity (3 pages). Ordering information is given on any current masthead page.

Registry No. Chemotactic protein methylesterase, 69552-31-4.

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Paradoxical Effects of Methylmercury on the Kinetics of Cytochrome *c* Oxidase†

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ABSTRACT: A stoichiometric amount of methylmercuric chloride substantially inhibits cytochrome *c* oxidase function under steady-state turnover conditions, where the enzyme is using its substrates, cytochrome *c* and oxygen, rapidly and continuously. Under these conditions, a reduction in activity of approximately 40% is observed. This is in accord with the results of Mann and Auer [Mann, A. J., & Auer, H. E. (1980) *J. Biol. Chem. 255*, 454–458], who used mercuric chloride and ethylmercuric chloride. Paradoxically, we found that addition of methylmercuric chloride can increase the activity of cytochrome *c* oxidase during its initial substrate utilization. This rate enhancement, measured under conditions where the enzyme cycles only a few times, is maximal for the resting state of the enzyme. "Pulsed" cytochrome *c* oxidase (i.e., enzyme that has been recently reduced and reoxidized) is considerably activated with respect to the resting enzyme, showing faster turnover rates (Antonini, 1977; Brunori et al., 1979). No significant rate enhancement upon treatment with methylmercuric chloride is seen in initial substrate utilization if the enzyme is pulsed immediately before the assay. The apparently contradictory effects of methylmercuric chloride on the resting and pulsed states of the oxidase under low turnover conditions may be reconciled by a model in which mercurial binding greatly stabilizes the enzyme in a state resembling that of the pulsed enzyme. A decrease in conformational flexibility may be the basis of the mercurial-induced diminution in activity of the enzyme during steady-state turnover conditions.

Many biological systems, e.g., enzymes, respiratory proteins, etc., have been reported to bind heavy metals. Binding of such metal atoms has also been shown to interfere with both the catalytic and regulatory functions of such systems. It is this binding that is presumably at the root of heavy-metal toxicity.

Cytochrome *c* oxidase, the terminal electron acceptor of the mitochondrial respiratory chain, plays a major role in cellular respiration. Organomercurials have no reported effects on the electron paramagnetic resonance (EPR) spectra of the enzyme (Beinert & Palmer, 1965). Mann & Auer (1980) have shown that the activity of cytochrome *c* oxidase drops to approximately 60% of its original value on binding a single atom of mercury per heme *a*, i.e., two Hg atoms per unit containing the full complement of four metal centers (two heme ions and two copper atoms). The activity of cytochrome *c* oxidase was reported to decrease sharply from the level of the control by addition of betwen 0 and 1 equiv of ethylmercuric chloride per heme *a*. Subsequent additions of ethylmercuric chloride up to 6 equiv per heme *a* had no further effect on the activity of the cytochrome *c* oxidase. The ethylmercury-modified enzyme maintains a relatively constant level of activity for at least several hours and, hence, is a stable modification (Mann & Auer, 1980). Mercury was shown to bind tightly to the enzyme and could not be removed by chromatographic techniques but only by thiol-exchange procedures. Because of its very high affinity for mercury, a sulfur-containing group almost certainly provides this mercury binding site (Mann & Auer, 1980; Darley-Usmar et al., 1981).

Sulfur atoms have been suggested to act as ligands for the copper atoms of cytochrome oxidase. For example, Blumberg & Peisach (1979) suggest that a copper atom liganded to sulfur is responsible for the EPR signal of the Cu center. Similarly, the amino acid sequence of subunit II shows homology with copper proteins (Steffens & Buse, 1979), particularly azurin and plastocyanin, again indicating that sulfur may act as a ligand to at least one of the copper atoms in this enzyme. In addition, Powers et al. (1981), on the basis of of EXAFS[1] data, proposed that in some states of the oxidized enzyme, sulfur may act as a bridging ligand between a copper atom (Cu_B) and the iron atom of cytochrome a_3, the oxygen binding site.

In order to explore further the mechanism of inhibition of cytochrome *c* oxidase by mercury, we have examined the effects of methylmercuric chloride (MeHgCl) on the activity

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; MeHgCl, methylmercuric chloride; EXAFS, extended X-ray absorbance fine structure.

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