Purification and characterization of protein methylase II from *Helicobacter pylori*

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Abstract

Protein methylase II (AdoMet:protein-carboxyl O-methyltransferase, EC 2.1.1.24) was identified and purified 115-fold from *Helicobacter pylori* through Q-Sepharose ion exchange column, AdoHcy-Sepharose 4B column, and Superdex 200 HR column chromatography using FPLC. The purified preparation showed two protein bands of about 78 kDa and 29 kDa molecular mass on SDS-PAGE. On non-denaturing gel electrophoresis, the enzyme migrated as a single band with a molecular mass of 410 kDa. In addition, MALDI-TOF-MS analysis and Superdex 200 HR column chromatography of the purified enzyme showed a major mass signal with molecular mass values of 425 kDa and 430 kDa, respectively. Therefore, the above results led us to suggest that protein methylase II purified from *H. pylori* is composed of four heterodimers with 425 kDa (4*(78+29) = 428 kDa). This magnitude of molecular mass is unusual for protein methylases II so far reported. The enzyme has an optimal pH of 6.0, a $K_m$ value of $5.0 \times 10^{-6}$ M for S-adenosyl-L-methionine and a $V_{max}$ of 205 pmol methyl$^{14}$C transferred min$^{-1}$ mg$^{-1}$ protein. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Purification; Protein methylase II; *Helicobacter pylori*

1. Introduction

Protein methylation occurs ubiquitously in nature, in organisms ranging from prokaryotic to eukaryotic, and involves $N$-methylation of arginine, lysine, histidine, alanine, proline, and glutamine, $O$-methylation of glutamic acid and aspartic acid, and $S$-methylation of cysteine and methionine [1-4]. The methyl donor for the reaction is $S$-adenosyl-$l$-methionine (AdoMet) and the demethylated product is $S$-adenosyl-$l$-homocysteine (AdoHcy).

Protein methylase II (EC 2.1.1.24), one of the carboxyl $O$-methylating enzymes, can methylate side-chain carboxyl groups of proteins with $S$-adenosyl-$l$-methionine [5-7]. The enzyme was first described by Axelrod and Daly in 1965 as a methanol forming enzyme in pituitary extracts [5], and subsequently Kim and Paik isolated a methyltransferase from calf thymus to catalyze carboxymethylation of proteins [6,8]. Protein methylase II has since been purified from various mammalian sources including brain, erythrocytes and testis [9-11]. In addition to mammalian sources, activities of protein methylase II have been detected in bacteria [12,13]. It is now clear that at least four distinct types of protein-carboxyl methyltransferases exist [7], and their suggested biochemical function includes bacterial chemotaxis [12], protein repair [13,14], and signal transduction [15]. However, the overall definitive physiological significance of protein methylase II remains to be clarified.

In 1984 Marshall and Warren discovered and succeeded in isolating *Helicobacter pylori* from stomach [18]. The first link between *H. pylori* and human disease was the
observation that this organism was almost invariably associated with chronic superficial gastritis [19]. In the absence of ulcer inducing medications, duodenal ulcer disease occurred almost exclusively among persons infected with *H. pylori*. In addition to the possible link of *H. pylori* to peptic ulcers, it was demonstrated that concurrent or previous *H. pylori* infection is associated with a 2.7-12-fold increased risk of gastric cancer [20]. Because gastric adenocarcinoma is one of the most common and deadly carcinomas worldwide, eradication or prevention of *H. pylori* infection might reduce the incidence of this cancer. In the present study, protein-carboxyl O-methyltransferase has been identified and purified from *H. pylori*. Unlike other protein-carboxyl O-methyltransferases, *H. pylori* protein methylase II has a distinct characteristic of molecular structure composed of four subunits of heterodimers, having a molecular mass of 425 kDa.

2. Materials and methods

2.1. Strains and culture conditions

*H. pylori* NCTC11637 was purchased from the National Collection of Type Cultures (NCTC, UK). *H. pylori* (Sydney strain) and clinically isolated *H. pylori* were kindly provided by Daewoong Pharmaceutical Co., Korea. *H. pylori* NCTC11637, *H. pylori* (Sydney strain), and clinically isolated *H. pylori* were cultivated by agar plate which was prepared using blood agar base No. 2 (Difco, USA) with antibiotics (vancomycin, bacitracin, amphotericin, polymyxin B, and nalidixic acid) with 10% blood [21]. Plates were incubated for 3 days at 37°C in 10% CO2.

2.2. Assay for protein methylase II

Enzyme activity was determined by a modified method of Kim and Paik [5]. The reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 0.6 mg of histone (calf thymus type II-As, Sigma Chemical Co., USA), and appropriate amounts of enzyme preparation in a total volume of 0.09 ml was preincubated at 37°C for 5 min, and was further incubated for 15 min after the addition of 0.01 ml of 25 μM Ado[methyl-14C]Met (130 dpm pmol⁻¹, Amersham Pharmacia Biotech, USA). At the end of incubation, 0.1 ml of 125 mM sodium borate buffer (pH 11.0) was added to the incubation mixture, and the mixture was further incubated for 5 min at 37°C. 1 ml of isoamyl alcohol was added to the mixture, the mixture was vortexed and centrifuged at 10 000×g for 5 min at room temperature, and the radioactivity of the supernatant (0.7 ml) was determined. One enzyme unit is defined as the amount of enzyme that catalyzes the transfer of 1 pmol methyl-14C to the methyl acceptor substrate min⁻¹ at pH 7.0 and 37°C. Specific activity is defined as units of enzyme per mg of protein. Protein concentration was determined by Bradford’s method [22], using bovine serum albumin as a standard.

2.3. Purification of protein methylase II from *H. pylori*

*H. pylori* collected from plates (wet weight 30 g) were suspended in four volumes of phosphate/phenylmethylsulfonyl fluoride (PMSF) buffer containing 0.1 M potassium phosphate, 1.0 mM EDTA, 0.1 mM PMSF, 5% glycerol and 0.24 mM β-mercaptoethanol (pH 7.0). The suspension was sonicated for 10 min, and the sonicate was centrifuged at 105 000×g for 1 h at 4°C. The cell-free extract was brought to 90% saturation with finely powdered ammonium sulfate. The suspension was stirred for 30 min and then centrifuged for 15 min at 105 000×g. The precipitate was resuspended in 10 ml buffer A (20 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, 0.1 mM EGTA, 0.24 mM β-mercaptoethanol, 0.1 mM PMSF, and 5% glycerol), dialyzed (10 kDa MW cut-off) against 4 l of buffer A overnight at 4°C, and applied to a Q-Sepharose column (i.d. 26 mm×10 cm, Amersham Pharmacia Biotech, USA). The enzyme was eluted with a linear gradient of buffer A containing 0–0.5 M NaCl at a flow rate of 1 ml min⁻¹. The active fractions having methylase activity were pooled and concentrated with ultrafiltration membrane (YM 10; cut-off of 10 kDa; Amicon, USA) to remove residual salt. The concentrate was subjected to an affinity column. AdoHcy-Sepharose 4B affinity column (i.d. 28 mm×3.4 cm), prepared by the method of Kim et al. [23], was equilibrated with buffer B (5 mM sodium phosphate, 5 mM EDTA, 2.4 mM β-mercaptoethanol, pH 6.2) at a flow rate of 1.8 ml min⁻¹. The enzyme was eluted with 0.5 mM AdoMet in buffer B. Fractions (1.8 ml) were collected into test tubes containing 0.2 ml of 50 mM sodium borate, 5% glycerol and 50 mM EDTA (pH 9.3) to
stabilize the eluted enzyme. The fractions eluted with AdoMet were concentrated by ultrafiltration membrane (YM 10; Amicon, USA) [23]. The above concentrated enzyme preparation was applied onto a Superdex 200 HR column (i.d. 1.0 U 30 cm, Amersham Pharmacia Biotech, USA) previously equilibrated with buffer A containing 0.15 M NaCl. The column was then eluted with buffer A at a flow rate of 0.4 ml min\(^{-1}\) (Fig. 1). The fractions containing protein methylase II activity were pooled, concentrated and residual salts were removed by ultrafiltration membrane (YM 10; Amicon, USA). The purified enzyme was stored at \(\sim 20^\circ\text{C}\) until use.

2.4. Estimation of molecular mass of the purified protein methylase II

The molecular mass of the purified protein methylase II was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE), non-denaturing PAGE, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Perseptive Analytical Elite, USA) analysis, and gel permeation chromatography. SDS-PAGE was performed according to the method of Laemmli [24] and non-denaturing PAGE was performed according to the method of Bhown and Bennett [25]. The molecular mass of the purified enzyme was determined using MALDI-TOF-MS. The purified protein methylase II from \(H.\ pylori\) was first dissolved in 0.1% trifluoroacetic acid, and then was added to the matrix solution (saturated dihydrobenzoic acid in 1:3:2 formic acid:water:2-propanol), so that the final protein concentration of the mixture was less than 0.1 mg ml\(^{-1}\). An aliquot of matrix/protein solution (0.5 µl) was then applied to a metal probe tip and dried [26,27]. Also, the molecular mass of the purified enzyme was determined by gel permeation on a Superdex 200 HR column. The relative elution volume of protein methylase II was compared with that of standard markers (vitamin B-12, 1.35 kDa; β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg ovalbumin (45 kDa), and carbonic anhydrase (29 kDa)).

2.5. N-terminal sequencing of the purified protein methylase II

The purified protein methylase II from \(H.\ pylori\) was subjected to SDS-PAGE and electroblotted on PVDF membrane. The membrane was dried, and the protein methylase II band was sequenced. N-terminal amino acid sequence analysis was performed with an Applied Biosystems Procise Protein Sequencer (Perkin-Elmer Applied Biosystems, USA), equipped with an on-line analyzer for phenylthiohydantoin-derivatized amino acids.

3. Results

3.1. Identification of protein methylase II from \(H.\ pylori\)

The presence of protein methylase II in three strains (NCTC11637, Sydney strain, clinical isolates) of \(H.\ pylori\) was identified. The specific activity of each strain was 0.5, 1.7, and 2.2 pmol Ado[methyl\(^{14}\text{C}\)]Met transferred min\(^{-1}\) mg\(^{-1}\) protein, respectively. Although the clinically isolated strain had the highest specific activity, it was very difficult to culture for the following experiment. Also, because

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg ml(^{-1}))</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Total activity (U a)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant after sonication</td>
<td>109</td>
<td>12.3</td>
<td>1341</td>
<td>1.6</td>
<td>2246</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td>88</td>
<td>13.8</td>
<td>1214</td>
<td>1.6</td>
<td>1942</td>
<td>86</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>37.5</td>
<td>3.8</td>
<td>143</td>
<td>6.3</td>
<td>901</td>
<td>40</td>
</tr>
<tr>
<td>AdoHcy-Sepharose 4B affinity</td>
<td>1.9</td>
<td>0.3</td>
<td>0.6</td>
<td>122</td>
<td>73</td>
<td>3.3</td>
</tr>
<tr>
<td>Superdex 200 HR gel permeation</td>
<td>1</td>
<td>0.04</td>
<td>0.04</td>
<td>184</td>
<td>7.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Data represent a typical purification procedure, which was repeated three times with similar results.

\(\text{aUnits: pmol methyl}^{14}\text{C transferred min}^{-1}\).

Fig. 2. Determination of molecular mass of the enzyme by SDS-PAGE and non-denaturing PAGE. A: M represents molecular mass standard markers including myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). A and G represent enzyme preparations purified by AdoHcy-Sepharose affinity and Superdex 200 HR column chromatography, respectively. B: M represents molecular mass standards used: apoferritin (443 kDa), urease (272 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and chicken egg ovalbumin (45 kDa). G represents enzyme preparation purified by Superdex 200 HR column chromatography.
strain NCTC11637 has a low specific activity, we selected the Sydney strain for purification of protein methylase II. As shown in Table 1, the enzyme was purified by Q-Sepharose ion exchange, AdoHcy-Sepharose 4B affinity and Superdex 200 HR column chromatography, and the final preparation had a specific activity of 184 pmol Ado[me-thyl-14C]Met transferred min\(^{-1}\) mg\(^{-1}\) protein with 115-fold purification and 0.3% yield.

3.2. Molecular mass and subunit size of protein methylase II from *H. pylori*

The purified protein methylase II preparation was resolved into two bands on SDS-PAGE, having molecular masses of 78 and 29 kDa (Fig. 2A). On the other hand, the enzyme migrated as a single band with a molecular mass of 410 kDa on non-denaturing gel electrophoresis (Fig. 2B).

The molecular mass of the purified enzyme preparation was also analyzed by Superdex 200 HR gel permeation and found to be approximately 430 kDa (data not shown), which was very close to the value of 410 kDa determined by non-denaturing gel electrophoresis (Fig. 2B). As shown in Fig. 3, MALDI-TOF-MS analysis of native protein methylase II showed a major mass signal with a molecular mass value of 425 073 Da.

3.3. N-terminal analyses of the purified protein methylase II from *H. pylori*

As shown in Table 2, N-terminal sequences of each subunit of protein methylase II from *H. pylori* were analyzed using the GenBank database system. The N-terminal sequence (1–12) of the 78-kDa subunit was similar to that of chlorohydrolase in strains 26695 and J99 of *H. pylori* with 91% identity, and the N-terminal sequence (1–20) of the 29-kDa subunit was similar to that of glutamine synthetase in strains 26695 and J99 of *H. pylori* with 91% identity, and the N-terminal sequence (1–20) of the

![Fig. 3. Analysis of protein methylase II by MALDI-TOF-MS.](image-url)
29-kDa subunit was similar to that of glutamine synthetase in strains 26695 and J99 of *H. pylori* with 80% and 75% identity, respectively. However, the molecular masses of chlorohydrolase (45.5 kDa) and glutamine synthetase (54.6 kDa) are significantly different from those of the subunits of purified protein methylase II. And there are no sequence homologies between these two enzymes and other protein methylases, indicating that protein methylase II purified from *H. pylori* is a novel enzyme, distinguished from chlorohydrolase, glutamine synthetase, and other protein methylases.

### 3.4. Characteristics of protein methylase II from *H. pylori*

There was a linear relationship between the amount of substrate protein (histone 0–64 mg) and enzyme activity (data not shown). The Michaelis constants \( K_m \) value and the maximum reaction velocity for AdoMet were determined by Lineweaver–Burk plot: \( K_m \) and \( V_{\max} \) values were \( 5.0 \times 10^{-6} \) M and 205 pmol min\(^{-1}\) mg\(^{-1}\) protein, respectively (Fig. 4). Table 3 shows that histone and hemoglobin served as the best in vitro substrates for the purified protein methylase II. The enzyme had optimum pH of 6.0 (data not shown).

![Fig. 4. Lineweaver–Burk plot of Ado(methyl-\textsuperscript{14}C)Met on protein methylase II from *H. pylori.*](image)

<table>
<thead>
<tr>
<th>Substrate protein</th>
<th>Specific activity (pmol min(^{-1}) mg(^{-1}))</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>174</td>
<td>97</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>152</td>
<td>84</td>
</tr>
<tr>
<td>Albumin</td>
<td>142</td>
<td>79</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>102</td>
<td>57</td>
</tr>
<tr>
<td>Gelatin</td>
<td>89</td>
<td>49</td>
</tr>
<tr>
<td>(\gamma)-Globulin</td>
<td>59</td>
<td>33</td>
</tr>
</tbody>
</table>

The incubation and assay conditions were as described in Section 2.

The amount of substrate used was 0.6 mg.

### 4. Discussion

Protein methylase II has been found in organisms ranging from prokaryotes to eukaryotes [1–4,28]. In the present study, the enzyme was purified from *H. pylori* by means of Q-Sepharose, AdoHcy-Sepharose 4B affinity and Superdex 200 HR chromatography. The final purified enzyme preparation had a specific activity of 184 pmol methyl-\textsuperscript{14}C min\(^{-1}\) mg\(^{-1}\) protein, representing a 115-fold purification with a yield of 0.3% (Table 1). This extent of purification is in the range of enzyme purifications from other sources: 1160-fold purification from *Escherichia coli* with a yield of 0.067% [12] and 13-fold purification from *Salmonella typhimurium* with 13% yield [14].

Purity and molecular mass of the purified enzyme was determined by SDS–PAGE, non-denaturing PAGE, MALDI-TOF-MS and gel permeation chromatography. The molecular mass of the enzyme determined by non-denaturing PAGE, MALDI-TOF-MS and gel permeation chromatography gave values of 410, 425, and 430 kDa, respectively. On SDS–PAGE, however, *H. pylori* protein methylase II was found to be composed of a heterodimer of 78 and 29 kDa (Fig. 2A). It is, therefore, highly likely that this enzyme appears to be composed of four heterodimers \((4 \times (78+29) = 428 \text{ kDa})\). The molecular mass of protein methylase II (AdoMet:protein-carboxyl O-methyltransferases) isolated from various organisms ranged from 24 to 33 kDa [6,8–11], however, the enzyme of *Streptomyces fradiae* comprised two identical subunits of 32 kDa [29]. Thus, the enormous size as well as the composition are quite unusual for protein methyltransferases in general. Among various substrates for *H. pylori* protein methylase II (Table 3), histone was found to be the most efficient substrate.
protein methylase II, showing no identity between protein methylase II from the Sydney strain of *H. pylori* and the other two enzymes. The discrepancy in molecular mass between protein methylase II purified from the Sydney strain of *H. pylori* and the other two enzymes deduced from GenBank data of strains 26695 and 399 of *H. pylori* might be attributed to a difference in genotype between strains. Also, similar sequences found in chlorohydrolase and glutamine synthetase have never been observed in other protein methylases. Therefore, protein methylase II from the Sydney strain of *H. pylori* appears to be a novel enzyme distinguished from other protein methylases.

The biological significance of the presence of protein methylase II in *H. pylori* is not obvious at present. However, the enzyme with the unusual characteristic of such a large molecular mass (425 kDa) with eight heterosubunits is suggestive of its complex function, and its function should be finely modulated in the stomach. Obviously, further studies on the exact amino acid sequence of protein methylase II of the Sydney strain of *H. pylori* will be needed in the stomach. Obviously, further studies on the exact amino acid sequence of protein methylase II, its location in the genome and the biological significance of *H. pylori* protein methylase II are needed.

5. Uncited references

[16,17]

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References