Purification and Characterization of Hen Oviduct Nα-Acetyltransferase*

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Hen oviduct Nα-acetyltransferase was purified to homogeneity by ammonium sulfate fractionation and DEAE-cellulose, Sepharose 6B, hydroxylapatite, and CoA affinity chromatography. The molecular weights of the native Nα-acetyltransferase and its protein subunit were estimated as 240,000 and 79,000, respectively. The purified enzyme exhibited a narrow pH optimum centered at 7.8. The enzyme was activated by diithiothreitol, cysteine, glutathione, and β-mercaptoethanol, but inhibited by Fe²⁺, Mn²⁺, Zn²⁺, Ca²⁺, Mg²⁺, and all thiol-specific reagents tested. These findings suggest that a thiol group(s) is essential to the enzyme activity.

Substrate specificity experiments of the purified enzyme revealed that (i) the minimal length of a peptide chain required for Nα-acetylation is 10 residues, (ii) the amino acids, Ala, Ser, Met, and Gly, which are predominantly found in the N termini of Nα-acetylated proteins, are not the sole determinant of Nα-acetylation for 10 and more residue peptides, and (iii) Nα-acetyltransferase recognizes a minute difference in the side chain structure at the N termini of ACTH₁₋₁₈-NH₂ and [Gly⁴]ACTH₁₋₁₈-NH₂, a productive and a nonproductive substrate, respectively.

In 1958 Narita (1) discovered an acetyl group at the N terminus of tobacco mosaic virus coat protein. Shortly after this discovery, Harris (2) also reported the presence of Nα-acetylserine at the N terminus of α-melanocite-stimulating hormone. Since then, a large number of Nα-acetylated proteins have been found widely in viruses, prokaryotes, and eukaryotes, and hence, Nα-acetylation is thought to be a mode of protein modification present universally in the nature. It was found that the amino acids susceptible to Nα-acetylation are mostly Ala, Ser, Met, and Gly. Furthermore, various ideas have been proposed as to a possible role(s) of Nα-acetylation. For instance, although the participation of Nα-acetylation in the initiation of protein biosynthesis has been concluded to be unlikely (3), the necessity in hormonal action (4), the enhanced oxygen affinity (5), and the protection from proteolytic degradation (6) and the involvement in protein secretion (7) are interesting so as to be thoroughly investigated.

Under these circumstances, it is of great importance to investigate the molecular mechanism of Nα-acetylation of proteins in order to understand its structural and functional roles in the biological world. Therefore, we have undertaken to isolate Nα-acetyltransferase from hen oviduct, since the enzyme was partially purified in our laboratory (8). In this paper, we report the purification of the enzyme to homogeneity and its enzymatic properties with respect to pH optimum, stability, activators and inhibitors, and substrate specificity.

EXPERIMENTAL PROCEDURES

RESULTS

Purification of Nα-Acetyltransferase—The enzyme was eluted in fraction III in the DEAE-cellulose chromatography (Fig. 1A) and near the void volume in the Sepharose 6B gel filtration, as expected (Fig. 1B). Two subsequent chromatographies were quite effective. The first was hydroxylapatite chromatography which could increase the purity of Nα-acetyltransferase up to 15-fold (Fig. 2). The second was the CoA affinity chromatography, by which the enzyme eluted with Ac-CoA (Fig. 3), became homogeneous (Fig. 4) and exhibited a high specific activity (193 units/mg). The result of purification was summarized in Table I. The enzyme was 2570-fold purified, and the yield was 1.32 mg from 500 g of hen oviducts.

The molecular weight of the Nα-acetyltransferase thus purified was estimated to be 240,000 by Sepharose 6B gel filtration (Fig. 5A) and 79,000 by SDS-polyacrylamide gel electrophoresis (Fig. 5B). This result indicates that the enzyme is an oligomeric protein composed of two or three subunits.

Enzymatic Properties—With ACTH₁₋₁₈, Nα-acetyltransferase was active in the narrow pH range centered at pH 7.8 (Fig. 6). The enzyme displayed a pH stability profile similar to the pH activity one (Fig. 7), but it was gradually inactivated at pH 7.8 at 25°C and higher temperatures (Fig. 8).

The enzyme was strongly inhibited by Fe²⁺, followed by Mn²⁺ and Zn²⁺ (Fig 9). Ca²⁺ and Mg²⁺ exhibited no effects on the enzymatic activity at concentrations as low as 0.1 mM, but, at higher concentrations, both ions were inhibitory. EDTA exhibited a peculiar behavior; the enzyme was 4-fold activated in the presence of 0.1 mM EDTA and completely inhibited at 2 mM and higher concentrations (data not shown).

From these results, the involvement of a thiol group(s) in the enzymatic activity was suggested. It was found that thiol compounds activated the enzyme significantly, for instance, up to 14-fold with 2 mM diithiothreitol (Fig. 10), whereas the enzyme was rapidly inactivated by the reaction with thiol-specific reagents such as mercuric dichloride, p-chloromercury-

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peptides bearing N-terminal Gly or Met, a potent candidate for protein N\textsuperscript{\textalpha}-acetylation. Under the present conditions, the Ser\textsuperscript{\textalpha} \rightarrow Gly replacement demolished the potentiality of ACTH\textsuperscript{1-18}-NH\textsubscript{2} as a substrate. Neither bovine insulin A-chain\textsuperscript{1-21} (Gly) nor methionyllysyl-bradykinin was N\textsuperscript{\textalpha}-acetylated, indicating that the potential amino acids such as Met and Gly are not the sole determinant of substrate specificity of N\textsuperscript{\textalpha}-acetyltransferase.

**DISCUSSION**

N\textsuperscript{\textalpha}-Acetyltransferase activity has been detected in the several sources such as Escherichia coli ribosomal protein L12 (13), rat liver ribosome-associated proteins (14-16), wheat germ ribosomal fraction (17), rabbit reticulocytes protein-synthesizing complex (9), calf lens (18), rat pituitary (19-21), and hen oviduct (8). Very recently an enzyme has been purified to homogeneity from yeast (22). The present work is the first successful purification of N\textsuperscript{\textalpha}-acetyltransferase of animal origin.

In our purification of N\textsuperscript{\textalpha}-acetyltransferase from hen oviduct, the stabilization of the enzyme was essential throughout the experimental procedures. Nikkol, a neutral nonionic detergent, and glycerol were effective. With 0.01% Nikkol and 5% glycerol, the enzyme retained 100 and 80%, respectively, of its activity at 4 °C for 1 month. Moreover, the use of CoA affinity chromatography and a newly devised 1-h assay method for N\textsuperscript{\textalpha}-acetyltransferase activity was very useful to shorten the duration of purification.

The molecular weight estimated for the native N\textsuperscript{\textalpha}-acetyltransferase was 240,000, which is in good agreement with the value reported previously (8). The numerical comparison between this value and that one estimated by SDS-polyacrylamide gel electrophoresis indicated that the enzyme consisted of three protein subunits of M\textsubscript{r} 80,000. Characterization of individual components and clarification of their assembly of this enzyme remain to be investigated.

N\textsuperscript{\textalpha}-Acetyltransferase is an acidic and hydrophobic enzyme but could not be eluted with 0.5% Tween 80, 0.5% Nikkol, or 0.01% SDS from hydrophobic columns involving phenyl, ω-aminopentyl, and Cibacron blue F3GA groups. The hydrophobic nature of the enzyme appears to be closely related to its subcellular localization.

An essential thiol group(s) was detected in N\textsuperscript{\textalpha}-acetyltransferase by chemical modification experiments with thiol compounds and thiol-specific reagents. The presence of a reactive thiol group(s) was confirmed by the fluorescence-staining of a single protein band with DACM on gel electrophoresis. Amino acid analysis of the performic acid-oxidized protein subunit also showed the presence of cysteic acids (data not shown). It is noteworthy that Ac-CoAarylamine N-acetyltransferases from pigeon (23), rat (24), and mouse (25) have shown). It is noteworthy that Ac-CoAarylamine N-acetyltransferases from pigeon (23), rat (24), and mouse (25) have essential thiol groups. However, the involvement of a histidine residue in the catalysis with hen’s enzyme cannot be ruled out, since histidine has been proposed to be the catalytic

<table>
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<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Purity</th>
<th>Relative activity</th>
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<tr>
<td>Extract</td>
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<td>2,270</td>
<td>100,000</td>
<td>7,530</td>
<td>1</td>
<td>100</td>
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<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} 30–70% precipitate</td>
<td>0.087</td>
<td>2,820</td>
<td>52,200</td>
<td>4,560</td>
<td>1.16</td>
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<td>DEAE fraction III</td>
<td>1.34</td>
<td>210</td>
<td>1,680</td>
<td>2,250</td>
<td>17.9</td>
<td>29.9</td>
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<td>Sepharose 6B No. 101-130</td>
<td>10.9</td>
<td>280</td>
<td>137</td>
<td>1,490</td>
<td>145</td>
<td>19.8</td>
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<td>166</td>
<td>37.5</td>
<td>3.59</td>
<td>596</td>
<td>2,210</td>
<td>7.92</td>
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<td>CoA affinity 0.5 mM Ac-CoA</td>
<td>193</td>
<td>12.5</td>
<td>1.32</td>
<td>235</td>
<td>2,570</td>
<td>3.39</td>
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</table>
Characterization of Hen Oviduct N\textsuperscript{\textalpha}-Acetyltransferase

The effects of divalent metal ions and EDTA on the enzymatic activity were complex. Nevertheless, it appears that Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, and Zn\textsuperscript{2+} that are inhibitory at 0.1 mM and higher concentrations to the enzyme might interact with a reactive thiol as does Hg\textsuperscript{2+}. The roles of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} which become inhibitory at concentrations higher than 0.1 mM are unclear. The concentration effect of EDTA is quite unusual. A likely explanation for this peculiar behavior of the enzyme to EDTA is a dual effect of the chelating agent. That is, the activation of the enzyme at 0.1 mM EDTA is due to removal of a metal ion(s) possibly bound to an essential thiol group(s). This coincides with the fact that thiol compounds are potent activators for the enzyme. The inactivation caused by higher concentrations over 0.1 mM of EDTA is supposed to be due to removal of the certain divalent metal ions essential to the generation of active conformation of the enzyme.

The present study showed that 10 residues are the minimal chain length for substrates of hen oviduct N\textsuperscript{\textalpha}-acetyltransferase and that yet unknown structural factors are additionally underlying to achieve substrate recognition and catalytic function. This result is in contrast to the report of Pease and Dixon (20), who showed that the minimal chain length of ACTH analogs for N\textsuperscript{\textalpha}-acetylation was 8 residues with rat pituitary N\textsuperscript{\textalpha}-acetyltransferase.

That, unlike ACTH\textsuperscript{1-18}-NH\textsubscript{2}, [Gly\textsuperscript{1}]ACTH\textsuperscript{1-18}-NH\textsubscript{2} is not a potent substrate for N\textsuperscript{\textalpha}-acetyltransferase is interesting, because the enzyme can recognize a subtle difference in the N-terminal structure of the ACTH molecule. Moreover, the decreased N\textsuperscript{\textalpha}-acetyltransferase activity for ACTH\textsuperscript{1-17} (4\%) relative to ACTH\textsuperscript{1-18}-NH\textsubscript{2} (52\%) suggests that Arg-18 or its amide plays a critical role. It is worthy to note that ACTH\textsuperscript{1-24} which has a cluster of basic residues at positions 18-24 is a more potent substrate than ACTH\textsuperscript{1-18}-NH\textsubscript{2} for N\textsuperscript{\textalpha}-acetyltransferase, an acidic enzyme.

Ovalbumin is a putative substrate of hen oviduct N\textsuperscript{\textalpha}-acetyltransferase. It is, therefore, interesting to consider our experimental results in conjunction with the in vitro synthesis of ovalbumin and subcellular localization of the enzyme. If we assume that the length of 10 or more residues and approximately 40 residues are necessary for N\textsuperscript{\textalpha}-acetyltransferase and penetration of the large subunit of ribosome (27-29), respectively, we can calculate 50 or more residues as the peptide chain length required for in vivo N\textsuperscript{\textalpha}-acetylation. This value is nearly equal to 50-60 residues of the nascent peptide chain which elongated long enough for the binding to microsomal membranes in vitro (30). If this assumption is correct, N\textsuperscript{\textalpha}-acetyltransferase should be situated at or near the region where the large subunit of ribosome is bound to the membrane of endoplasmic reticulum. This is compatible with the finding that N\textsuperscript{\textalpha}-acetyltransferase activity was recovered in the rough microsomal fraction (yield, 45\% 2.2-fold enrichment of the enzyme over the hen oviduct homogenate), and the enzyme is located on the cytoplasmic surface of rough endoplasmic reticulum (31).

Acknowledgments—We thank Dr. Katsuyoshi Mihara (Department of Molecular Biology, Graduate School of Medical Science, Kyushu University) for his generous advice and many valuable suggestions. We are also grateful to Dr. Ken Inouye (Shionogi Pharmaceutical Co., Ltd.) for the gifts of ACTH\textsuperscript{1-18}-NH\textsubscript{2} and [Gly\textsuperscript{1}] ACTH\textsuperscript{1-18}-NH\textsubscript{2} and Dr. Takeharu Masaki (Faculty of Agriculture, Ibaraki University) for the gift of Actinobacter protease I.

**TABLE II**

<table>
<thead>
<tr>
<th>Acetyl-acceptor</th>
<th>NH\textsubscript{2}-terminal Extent of N\textsuperscript{\textalpha}-acetylation*</th>
<th>10 amino acids</th>
<th>%</th>
</tr>
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<tr>
<td>ACTH\textsuperscript{1-5}</td>
<td>SYME</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ACTH\textsuperscript{1-8}</td>
<td>SYMEMHR</td>
<td>0</td>
<td></td>
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<tr>
<td>ACTH\textsuperscript{1-10}</td>
<td>SYMEMHRWG</td>
<td>4.7</td>
<td></td>
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<tr>
<td>ACTH\textsuperscript{1-11}</td>
<td>SYMEMHRWG</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>ACTH\textsuperscript{1-17}</td>
<td>SYMEMHRWG</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>ACTH\textsuperscript{1-18}-NH\textsubscript{2}</td>
<td>SYMEMHRWG</td>
<td>52</td>
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</tr>
<tr>
<td>ACTH\textsuperscript{1-24}</td>
<td>SYMEMHRWG</td>
<td>100</td>
<td></td>
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<tr>
<td>ACTH\textsuperscript{1-29}</td>
<td>SYMEMHRWG</td>
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<td>ACTH\textsuperscript{1-40}</td>
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<td></td>
</tr>
<tr>
<td>ACTH\textsuperscript{1-54}</td>
<td>HFRWG</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>[Gly\textsuperscript{1}]ACTH\textsuperscript{1-18}-NH\textsubscript{2}</td>
<td>SYSME</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>[Gly\textsuperscript{1}, Leu\textsuperscript{3}]Ovl\textsuperscript{1-9}</td>
<td>GSIGAASL</td>
<td>0</td>
<td></td>
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<tr>
<td>OMe\textsuperscript{8}</td>
<td>YGGFLKIRP</td>
<td>0</td>
<td></td>
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<tr>
<td>Dynorphin\textsuperscript{1-13}</td>
<td>DRRVIHPFHL</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Angiotensin I\textsuperscript{1-10}</td>
<td>GIVEOCKCTS</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Insulin A-chain\textsuperscript{1-21}</td>
<td>MKRPFGPF</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Methionyllysyl-bradykinin\textsuperscript{1-21}</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*: N\textsuperscript{\textalpha}-Acetyltransferase activity was assayed using 10 nmol of substrate as described under "Experimental Procedures." The extent of N\textsuperscript{\textalpha}-acetylation was expressed by relative values to 100\% of ACTH\textsuperscript{1-24}.

\textsuperscript{8} Ov, ovalbumin; OMe, methylester.
REFERENCES

Characterization of Hen Oviduct N\textsuperscript{\textalpha}-Acetyltransferase

Preparation of Hen Oviduct N\textsuperscript{\textalpha}-Acetyltransferase. One milligram of N\textsuperscript{\textalpha}-Acetyltransferase was renatured in 7 M urea at 37°C with Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}, 20 mM Tris-HCl (pH 8.0) in 500 μl of 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 100 mM NaCl, and 6 M urea, 20 mM Tris-HCl (pH 8.0) for 5 h. The solution was then dialyzed against 20 mM Tris-HCl (pH 8.0) for 12 h. Each digest of N\textsuperscript{\textalpha}-Acetyltransferase was separated by reverse-phase HPLC using linear gradients of 10 to 40% 2-propanol. The fractions were analyzed for their amino acid compositions with a Hitachi 850 amino acid analyzer. The presence of N\textsuperscript{\textalpha}-Acetyltransferase was detected with a Bio-Rad protein assay kit. The results showed that the purified enzyme is one acetyltransferase that acetylates the N\textsuperscript{\textalpha}-terminal group of N\textsuperscript{\textalpha}-Acetyltransferase, as described in "Experimental Procedures." The results showed that the purified enzyme is one acetyltransferase that acetylates the N\textsuperscript{\textalpha}-terminal group of N\textsuperscript{\textalpha}-Acetyltransferase, as described in "Experimental Procedures." The results showed that the purified enzyme is one acetyltransferase that acetylates the N\textsuperscript{\textalpha}-terminal group of N\textsuperscript{\textalpha}-Acetyltransferase, as described in "Experimental Procedures."
Characterization of Hen Oviduct Nα-Acetyltransferase

Fig. 8  Heat sensitivity of Nα-acetyltransferase. Nα-Acetyltransferase (protein 10 μg) was preincubated at 4°C ( ), 5°C ( ●), 37°C ( △), or 55°C ( ▽). Aliquots (protein 2 μg) of the enzyme solution were removed at the designated times and assayed under the standard conditions as described in "Experimental Procedures".

Fig. 10  Effects of thiol compounds on Nα-acetyltransferase activity. Nα-Acetyltransferase (protein 1 μg) was preincubated at 4°C for 5 min with DTT ( △), NεGly ( ■), NεGly ( △), or FeCl3 ( ▽) at the indicated concentrations. The enzyme activity was assayed under the standard conditions as described in "Experimental Procedures".

Fig. 12  SDS-polyacrylamide gel electrophoresis of BACM-treated Nα-acetyltransferase. Nα-Acetyltransferase (protein 17 μg) treated with BACM was dialyzed against water and then lyophilized. The lyophilate was subjected to SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn (11) using a 10% (w/v) acrylamide gel. The material in the radioactive peak at the retention time of 15.6 min (shown by a triangle) was pooled. The black boxes in the figure indicate radioactivity.

Fig. 13  Detection and isolation of [1-3H]Ac-ACTH1-24 by reverse-phase HPLC. (A) After the acetylation reaction had been carried out at 37°C for 3 h in a 10-fold scale of the standard enzyme assay mixture, 50 μl of the mixture was analyzed on a TSK gel ODS-120A column (4.6 x 250 mm). Elution was performed first with 20% acetonitrile for 10 min and then with a linear gradient of 20 to 50% acetonitrile for 20 min. The radioactive [1-3H]Ac-ACTH1-24 and the cold Ac-ACTH1-24 (1:74) eluted at 20.6 min in Fig. 13A, the mixture was rechromatographed with a linear gradient of 10 to 50% acetonitrile for 30 min. The material in the radioactive peak at the retention time of 15.6 min (shown by a triangle) was pooled. The black boxes in the figure indicate radioactivity.