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IMMUNOREACTIVITY OF RECOMBINANT HUMAN ADP-RIBOSYLARGININE HYDROLASE

Takashi SHINGU[b], Nobumasa HARA[a], Harumi OSAGO[a], Masaharu TERASHIMA[a], Kazuo YAMADA[a], Toshiki YAMASAKI[b], Kouzo MORITAKE[b], Makoto SHIMOYAMA and Mikako TSUCHIYA[a]

Departments of [a]Biochemistry and [b]Neurosurgery, Shimane Medical University, Izumo 693-8501, Japan

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ADP-ribosylarginine hydrolase (AAH) has not been detected yet in human tissues with anti-AAH antibodies. Those failure in human AAH (hAAH) detection are possibly because hAAH is not recognized with the only available antibodies, rabbit anti-rat AAH (rAAH) polyclonal antibodies or amounts of AAH in human tissues are too small to be detected with the antibodies. To clarify the interaction between hAAH and anti-rAAH antibodies, purified authentic hAAH is necessary. In this study, the protein was expressed as a histidine-tagged recombinant protein in *Escherichia coli* and purified to apparent homogeneity by a metal chelation chromatography. The purified protein, similar in properties to hAAH reported previously, catalyzed the glycohydrolysis of ADP-ribosylarginine. On Western blotting, the anti-rAAH antibodies interacted with hAAH, but not so strongly as with the rat hydrolase. Thus, anti-hAAH antibodies are needed to detect trace amounts of the antigen in human tissues.

Key words: human ADP-ribosylarginine hydrolase, immunoreactivity, purification

INTRODUCTION

Arginine-specific ADP-ribosylation is a post-translational modification of proteins, catalyzed by arginine-specific ADP-ribosyltransferase, transferring the ADP-ribose moiety from NAD to arginine residues in cellular proteins (1). Another enzyme, ADP-ribosylarginine hydrolase (AAH) attacks the α-anomer of ADP-ribosylarginine and releases ADP-ribose from the arginine. It has been considered that arginine-specific ADP-ribosyltransferase and AAH could catalyze the opposing arms of an ADP-ribosylation-deADP-ribosylation cycle in which arginine is modified and regenerated and that the cycle could serve as a reversible regulatory mechanism of protein functions (2). For example, in nitrogen fixing bacteria *Rhodospirillum rubrum*, ADP-ribosylation-deADP-ribosylation of a specific arginine residue of the dinitrogenase reductase has been shown to regulate the reductase activity (3). In eucaryotes, ADP-ribosyltransferase and AAH have been found in various tissues and cells (4), and some of them were purified and cloned (4, 5). However, the endogenous target protein of this potential regulatory cycle and the precise role of modification in animal tissues remain to be clarified.

Previously, we expressed a recombinant rat AAH (rAAH) protein in *E. coli*, and immunized rabbits with the AAH to generate anti-AAH antibodies. Using the antibodies, which recognize AAH protein in rat and mouse brains, we described the distribution of AAH in rodent central nervous system and suggested some possible functions of the enzyme as well as the ADP-ribosylation-deADP-ribosylation cycle (6). In human brain, however, we have not yet detected the AAH successfully using the anti-rAAH antibodies. Possible reasons for failure in human AAH (hAAH) detection are thought that hAAH is not recognized with anti-rAAH antibodies or that the quantity of hAAH expression is too small to be visualized on immunoblotting. In the previous study using a recombinant hAAH, Takada *et al.* postulated that the conformation of hAAH may be different from that of rAAH (5). However, the recombinant protein used in their experiments was not completely purified and their assumption is not very feasible since the identity of the amino acid sequences between two proteins is more than 80% (5). To define whether hAAH as well as

Correspondence: Mikako Tsuchiya, Shimane Medical University, Izumo 693-8501, Japan. Fax: 853-20-2120.

rAAH is interacted with anti-rAAH antibodies or not, highly purified authentic hAAH is indispensable.

In the present study using a histidine-tagged protein expression system, we purified recombinant hAAH, examined the immunoreactivity of the hAAH to anti-rAAH polyclonal antibodies and revealed that even though with lower affinity than rAAH, hAAH could be recognized by the antibodies.

MATERIALS AND METHODS

Preparation of recombinant hAAH

cDNA was prepared from human white blood cell total RNA (10 μg) using reverse transcriptase (Perkin-Elmer-Cetus) and random hexamers. Polymerase chain reactions (PCRs) with two different polymerases were done for 35 cycles of amplification followed by 7 min of extension. A PCR cycle with *delta-Tth* DNA polymerase (Toyobo) consisted of heating at 93℃ for 35 s, annealing at indicated temperatures in the parentheses for 35 s, and extension at 72℃ for 2 min. For *AmpliTaq* DNA polymerase (Perkin-Elmer-Cetus) amplification, reaction mixture was incubated at 95℃ for 1 min and then at the temperatures indicated in the parentheses in a cycle.

Four segments of hAAH cDNA were amplified from the cDNA template in separate reactions with combinations of primers F3 (GGCCTGGTTCCGCGGGAGGGACTGATGGAG) and R6 (GCAGTCTTGGTAATGCTTAGCAAGGAGGT), F6 (ACCTCCTTGCTAAGCATTACCAAGACTGC) and R4 (GCTTCTGGTAGCAGCTCCATCAGTCC), F2 (CTGATCCAAGTGAGCATC) and R2 (GAATCACTGTCTCCACC), and F5 (CAACACTGGTCCTACTTC) and R3 (CTGCGCCTC-GCTCCGAACATCACGTCTCC) respectively, the former two of which were amplified with *delta-Tth* DNA polymerase (63℃) and the latter two with *AmpliTaq* DNA polymerase (60℃). The segments resulting from primers F2-R2 and F5-R3 were combined and amplified with *delta-Tth* and primers F2 and R3 (60℃). The product was combined with the segment from F6-R4 and amplified with *delta-Tth* and primers F6 and R3 (50℃). The product was combined with the segment from F3-R6 and amplified with *AmpliTaq* and primers F3 and R3 (60℃). To the fragment containing full length of the hAAH coding region *Bam* HI sites were provided by amplification with *AmpliTaq* (68℃) and primers HAAHF15b2 (GGTACCGGATCCGGAGAAGTATGTGGCTGC) and HARHR15b (GGTACCGGATCCCTAAAGGGAAATTACAGT).

The product was digested with *Bam* HI (New England Biolabs), ligated to *Bam* HI-digested pET15b vector (Novagen) and transfected into *E. coli* DH5α cells (Takara). After ampicillin selection for transfected cells, the plasmid containing hAAH cDNA was purified, sequenced and further transfected to *E. coli* BL21 (DE3). Histidine-tagged hAAH (his_6-hAAH) was expressed in the cells with induction at 28℃ for 3 h with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) in 1000 ml culture. Cells were harvested and suspended in 20 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 8.0) followed by sonication. Sixteen ml of binding buffer and 4 ml of 10% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were further added and the mixture was stirred for 2 h at 4℃ and centrifuged at 39,000 x g for 20 min. According to the manufacturer's protocol, the resultant supernatant was applied to the *His-Bind* metal chelation resin (Novagen) on which Ni^{2+} was immobilized, and after unbound proteins were washed away, the recombinant protein was eluted with 1 M imidazole. The protein was eluted somewhat with other proteins in wash buffer which contained 60 mM imidazole (Fig. 1, lane 6).

AAH assay

Purified his_6-hAAH (0.5 μg) and ADP-ribosyl [^{14}C]arginine (16,000 dpm/nmol, 36 μM) were incubated at 37℃ for 30 min in 50 mM Tris-Cl buffer (pH 7.5) containing 5 mM dithiothreitol (DTT) with or without 10 mM $MgCl_2$ in a total volume of 50 μl. After the incubation, 250 μl of 0.1% trifluoroacetic acid (TFA) was added to stop the reaction and 200 μl of the mixture was subjected to a reversed-phase C18 column (5C18-MS, Nacalai Tesque) and developed with 0.1% TFA. The eluate from the column was collected in 0.5 ml fractions and the radioactivity in 250 μl of each fraction was counted (7).

Immunoreactivity of his_6-hAAH and recombinant glutathione S-transferase (GST)-rAAH fusion protein

GST-rAAH and the antibodies against the recombinant protein were prepared as described previously (6, 7). his_6-hAAH and GST-rAAH were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) in 15% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with immunoaffinity-purified rabbit anti-rAAH polyclonal antibodies as described previously (6).

RESULTS AND DISCUSSION

An *E. coli* strain BL21(DE3) was transfected with a plasmid for histidine-tagged expression of the coding region of hAAH, and grown. The bacterial cells were further incubated either with or without IPTG for 3 h. In the presence of IPTG, the density of a band, a protein with a molecular mass of 40～41 kDa consistent with his_6-hAAH in size, appeared in the bacterial homogenate (Fig. 1, lanes 1 and 2). After treatment of the homogenate with CHAPS, considerable amount of the protein appeared in the supernatant fraction. After a metal chelation chromatography, the protein from the supernatant was purified to apparent homogeneity.

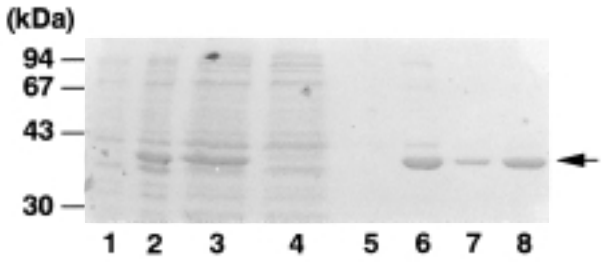

Fig. 1. Purification of recombinant human ADP-ribosylarginine hydrolase. Samples from the cell lysates before (1) and after IPTG-induction (2), and from the fractions of CHAPS supernatant (3), pass-through (4), wash I (5), wash II (6), eluate I (7) and eluate II (8), from the metal chelation column, were subjected to SDS/PAGE and stained as described under Methods. The positions of his_6-hAAH (arrow) and protein standards are indicated.

To confirm AAH activity of the purified protein, we incubated a mixture of ADP-ribosyl[^{14}C]arginine anomers, the substrate of the AAH, with the recombinant protein and analysed the reaction products by HPLC. In the presence of Mg^{2+}, the α-anomer of ADP-ribosylarginine decreased and [^{14}C] arginine appeared, while [^{14}C]arginine was not detected when Mg^{2+} was omitted from the reaction mixture (Fig. 2). These observations indicate thatthe purified protein catalyzes the cleavage of the α-ADP-ribosylarginine anomer to ADP-ribose and arginine, in a Mg^{2+}-dependent manner. We also examined the effect of DTT on the activity and observed that the removal of DTT from the reaction mixture did not decrease the AAH activity of the purified protein (data not shown). The characters of the purified protein shown in these experiments were consistent with those of hAAH in the previous report (5) and we concluded that the authentic hAAH was obtained. The specific activity of the recombinant hydrolase was 102 nmol/min/mg, which was approximately twice of that of the recombinant hydrolase reported by Takada *et al.* (5).

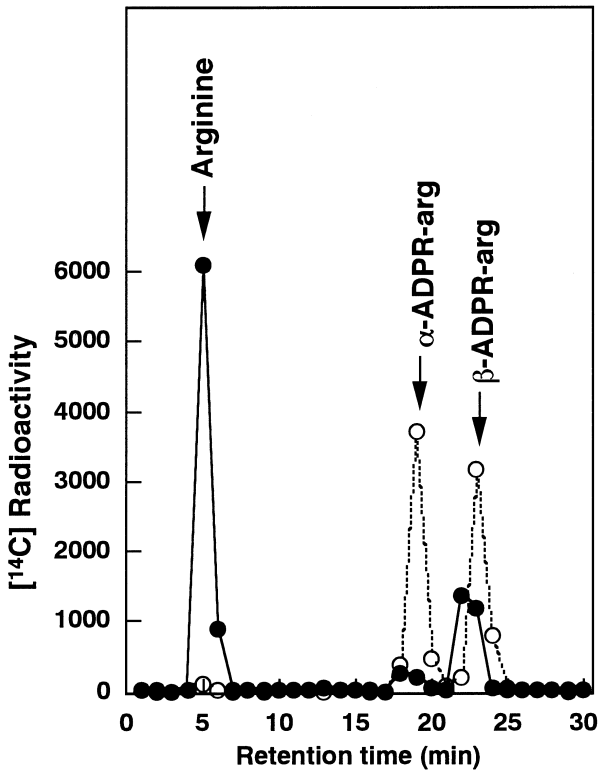

Fig. 2. Cleavage of the ADP-ribosylarginine bond by his_6-hAAH. A mixture of ADP-ribosyl[^{14}C]arginine anomers was incubated with his_6-hAAH in the presence (closed) or absence (open) of Mg^{2+}. After the incubation, the reaction mixture was applied to a reversed-phase C18 column and the radioactivity in the eluate from the column was counted. α- and β-ADPR-arg indicate α- and β-anomers of ADP-ribosylarginine, respectively.

Immunoreactivity of the recombinant hAAH was examined with affinity-purified rabbit anti-rAAH polyclonal antibodies. As shown in Fig. 3, on Western blotting, the antibodies detected not only rat (GST-rAAH, 65 kDa), but also human (his_6-hAAH) AAH. Even though the amount of his_6-hAAH subjected to the analysis was similar to that of GST-rAAH based on SDS/PAGE and staining, the density of the immunoreactive band of his_6-hAAH on the blot was lower than that of GST-rAAH. These results indicate that the antibodies do bind to his_6-hAAH, with an affinity lower than that to GST-rAAH.

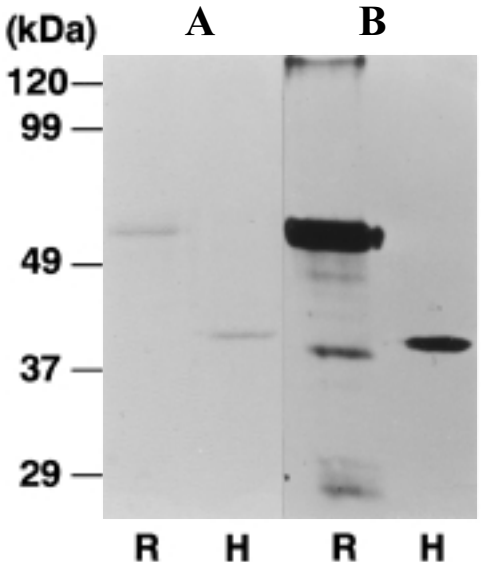

Fig. 3. Immunoreactivity of human and rat AAH. his_6-hAAH (H, 5 μg) and GST- rAAH (R, 5 μg) were subjected to SDS/PAGE, then stained (A) or blotted and probed with anti-rAAH polyclonal antibodies (B). The positions of protein standards are indicated.

Takada *et al*. examined the immunoreactivity of hAAH with anti-rAAH antibodies using partially purified hAAH preparation and reported that the antibodies did not react with the hAAH, suggesting that the conformation of hAAH is considerably different from those of rodent AAHs (5). However, in our experiments, the hAAH purified to apparent homogeneity was recognized by rabbit anti-rAAH antibodies, though the reactivity was lower than that of rAAH. The result is well consistent with the fact that hAAH is 83% identical in amino acid sequences to rAAH (5). Thus we conclude that the anti-rAAH antibodies would not detect the antigen in human tissues not because of different conformation of hAAH but owing to its lower affinity, together with the lower content of AAH in human tissues (5). Therefore, we are preparing rabbit anti-hAAH polyclonal antibodies with a titer high enough to detect such small amounts of hAAH on immunoblotting and immunohistochemical studies of human tissues.

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