Monoclonal antibodies to renal brush border membrane maltase: Age-associated antigenic alterations  

(aging/ altered enzyme/ rat kidney disaccharidase)

Uzi Reiss and Bertram Sacktor

Laboratory of Molecular Aging, National Institutes on Aging, National Institutes of Health, Gerontology Research Center, Baltimore City Hospitals, Baltimore, Maryland 21224

Communicated by R. H. Wasserman, February 28, 1983

ABSTRACT  Monoclonal antibodies to maltase (a-d-glucoside glucohydrolase, EC 3.2.1.20) from young adult and aged rats were prepared by the hybridoma technique. Four cell lines producing antibodies of the IgG1 subclass to maltase were established. Two, designated 1F12E1 and 9B1G6, produced monoclonal antibodies specific for the catalytically active form of the enzyme found predominantnly in enzyme preparations from young animals. The other two clones designated 7G10H3 and 2E1C10 produced monoclonal antibodies that reacted exclusively with an enzymatically inactive form of maltase found mostly in enzyme preparations from aged rats. The increased prevalence of an inactive form of the enzyme in the old rat accounts for the decreased maltase-specific activity previously reported in the senescent rat. The active and inactive maltase species were separated by immunoaffinity chromatography by using the monoclonal antibodies as ligands. The separated forms of the enzyme were not distinguished by NaDodSO4/polyacrylamide gel electrophoresis, peptide mapping of the CNBr-cleaved proteins, and the NH2-terminal residues of these peptides. This study demonstrates the presence of an altered, antigenically distinct enzyme in senescent animals. Critical issues on the mechanism of the aging process may be addressed by application of these findings.

Maltase (a-d-glucoside glucohydrolase, EC 3.2.1.20) is the major disaccharidase in rat kidney brush border membranes (1, 2). Maltase-specific activities in renal cortex homogenates and brush border membranes prepared from aged rats decrease 30-40% relative to the activities of the enzyme from young adult animals (3, 4). When the enzyme from senescent and mature rats is solubilized and purified to homogeneity, the same decrement with age is found (5). This finding indicates that the decrease in maltase activity with age results from an alteration in the enzyme per se, rather than from a change in the enzyme’s membrane environment, which is reflected secondarily in a loss in activity. The pure “young” and “old” enzymes do not differ measurably in molecular weight, electrophoretic mobility, and amino acid composition. However, significant differences are found in their circular dichroism spectra, perhaps suggesting a conformational alteration with age (5). These results prompted us to examine whether this age-dependent molecular change is associated with modification in the antigenicity of the enzyme. The present communication shows that monoclonal antibodies (MAbs) specific to young and old forms of maltase can be obtained by hybridoma technology and that the two species of enzyme can be separated by using the MAbs as immunoaffinity ligands. Thus, we demonstrate the presence of an altered, antigenically distinct enzyme in senescent animals.

MATERIALS AND METHODS

Enzyme Purification. Kidney brush border maltase was purified to homogeneity from mature (6 mo) and aged (24 mo) female Wistar-derived rats as described (5, 6). The animals were obtained from the Animal Resources Facility, Gerontology Research Center, National Institute on Aging. (The Animal Resources Facility, Gerontology Research Center, is fully accredited by the American Association for Accreditation of Laboratory Animal Care.) Maltase activity was determined by a coupled enzymatic assay (2).

Iodination of Maltase. Pure maltase (200 ym of protein in 0.5 ml of 0.01 M Na phosphate, pH 7.4) from young or old animals was iodinated by a lactoperoxidase procedure (7). The enzyme was incubated at room temperature for 15 min with 5 yl of Na125I (100 mCi/ml, Amersham; 1 Ci = 3.7 x 1010 Bq), 10 yl of lactoperoxidase (0.3 mg/ml, Sigma), and 10 yl of H2O2 (10 yM). After incubation, the reaction mixture was chromatographed on a Sepharose 4B/Tris affinity column to which maltase was selectively bound (6). The labeled maltase fractions were combined and then dialyzed. In some experiments the iodinated enzyme was chromatographed on a Sepharose 4B column (6) rather than on the affinity column.

Preparation of MAbs. MAbs to maltase from young and old rats were prepared by the hybridoma technique (8). BALB/c female mice were immunized by intraperitoneal injection of a mixture of pure “young” and “old” maltase (150 ym of enzyme protein of each) in 0.2 ml of phosphate-buffered saline (10 mM Na phosphate, pH 7.2/150 mM NaCl), together with 0.2 ml of complete Freund’s adjuvant. Two additional injections, without adjuvant, were given intraperitoneally at 3-wk intervals. Four days after the last injection, the spleens were removed, and cells were prepared by rubbing the tissue on a 45 x 45 mesh screen. Approximately 106 spleen cells were fused with 105 myeloma cells (x63-Ag 8.6.5.3) (9). The cells, about 105 spleen and 106 myeloma cells per well, were grown in 96-well Costar plates in selective hypoxanthine/aminopterin/thymidine medium (9). At 2–3 wk after fusion the surviving hybrids were tested for production of specific anti-maltase antibodies by the enzyme-linked immunosorbent assay (ELISA) technique (10). In the initial ELISA screening procedure, a mixture of the maltase (1 ym of enzyme protein of each) from adult and aged rats served as the antigen. Positive cultures were tested for reactivity with young and old maltase separately and were then cloned by limiting dilutions. The resulting MAb-generating cells (103 cells in 1 ml of phosphate-buffered saline) were injected into Pristane (0.5-ml)-treated BALB/c mice to obtain increased production.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody.

*To whom reprint requests should be addressed.
of antibodies in the ascites fluid. The MABs in the ascites fluid were either used directly, or the IgG in the fluid was first purified by affinity chromatography on a protein A-Sepharose 4B column. In three fusion experiments, 20–30% of the wells contained hybrids. Of these, 1–13% produced antibodies that were positive against maltase. A total of four cultures were established that were stable producers of anti-maltase antibodies.

Affinity Binding of Maltase to MAB-Protein A-Sepharose 4B. Maltase affinity columns were prepared by slowly adding MAB-containing ascites fluid brought to pH 8.0 with Na phosphate buffer (final concentration, 0.1 M) onto protein A-Sepharose 4B columns (Pharmacia) at room temperature. Each column was washed with the 0.1 M phosphate buffer (pH 8.0) to remove unbound maltase. Maltase, either 125I-labeled or unlabeled, was placed on the column at room temperature. After being washed with the phosphate buffer, the MAb–enzyme complex was eluted with 0.1 M acetic acid or 0.1 M citrate (pH 6.0). The eluant was either neutralized with NH4OH and lyophilized or dialyzed against 0.01 M Na phosphate buffer (pH 8.0). Radioactivity was counted in a gamma counter.

Immunoprecipitation of Maltase Activity with MABs. Maltase (0.12 units) from mature and aged rats was incubated overnight at 4°C with different dilutions of MAB-containing ascites fluid in a total volume of 0.2 ml of 0.01 M Na phosphate buffer (pH 7.4). The following morning, the incubation medium was adjusted to pH 8.0, and the ionic strength was raised to 0.1 M by the addition of 0.5 M Na phosphate (pH 8.0). Then, 0.2 ml of a 50% suspension of protein A-Sepharose 4B beads in 0.1 M Na phosphate buffer (pH 8.0) was added. The mixture was rotated at room temperature for 1 hr, centrifuged, and the maltase activity remaining in the supernatant was determined.

NaDodSO4/Gel Electrophoresis. The maltase eluted from the MAB–protein A-Sepharose 4B affinity column was incubated with 0.01 M Na phosphate, pH 7.0/1% NaDodSO4/1% mercaptoethanol for either 2 hr at 37°C or overnight at room temperature. The enzyme was electrophoresed on a 3.75% acrylamide slab gel with Tris/glycine/1% NaDodSO4, pH 8.4, and a current of 200 mA. The gels were stained for protein with Coomassie blue or dried under vacuum for autoradiography. Kodak XAR-5 x-ray film was used.

CNBr Cleavage and Peptide Mapping. Cleavage of maltase with CNBr and dansylation of the resultant peptides were carried out as described (12, 13). The CNBr-cleaved peptides were mapped by applying approximately 50 μg of protein to 20 × 20 cm cellulose-coated thin-layer plates, followed by electrophoresis in pyridine/acetic acid/water, 1:10:90 (vol/vol), pH 3.7, for 40 min at room temperature with 40-nA current. Chromatography was carried out with 1-butanol/pyridine/acetic acid/water, 50:33:1:40 (vol/vol). The plates were dried and sprayed with ninhydrin reagent.

RESULTS

Four MABs to maltase, produced by clones designated IF12E1, 8B1G6, 7G10H3, and 2E1C10, were obtained. They were of the IgG1 subclass, as determined by immunodiffusion with rabbit anti-mouse IgG antibodies; were bound specifically to proximal tubular cell brush border membranes, as detected by immunofluorescence; and did not inhibit maltase activity when bound to the enzyme.

Fig. 1 shows titration curves for the binding of MAB 8B1G6 and MAB 7G10H3 to pure maltase from mature and aged rats when either equal maltase activity (0.025 unit; Fig. 1 A and C) or equal enzyme protein (1 μg; Fig. 1 B and D) was applied to ELISA plates, and the amount of IgG bound was estimated by a coupled reaction with horseradish peroxidase-conjugated rab-

![Fig. 1. The binding of MABs synthesized by clones 8B1G6 (A and B) and 7G10H3 (C and D) to pure maltase from 6-mo-old (●) and 24-mo-old (●) rats, determined by the ELISA assay in which the IgG bound was estimated by a coupled reaction with horseradish peroxidase-conjugated rabbit anti-mouse IgG. In A and C, equal maltase activities (0.025 unit) were applied to the plates. In B and D equal enzyme proteins (1 μg) were applied.](image1)

![Fig. 2. The precipitation of maltase activity with MABs synthesized by clones IF12E1 and 7G10H3. Maltase preparations from young (●) and aged (●) rats (0.12 unit of activity) were incubated with the indicated amounts of antibody. To facilitate the precipitation of the enzyme–antibody complex, a suspension of protein A-Sepharose 4B beads was added to each incubation, and the mixture was rotated for an additional 1 hr at room temperature. After centrifugation, the maltase activity remaining in the supernatant was determined.](image2)
the young and old enzyme preparations (Fig. 2). In contradistinction, MAb 7G10H3, although it was capable of binding the maltase protein from aged animals (Fig. 1), did not immunoprecipitate activity when incubated with enzyme preparations from old or young rats. In fact, essentially 100% of the enzyme activity remained in the supernatant.

These results (Figs. 1 and 2) suggested that MAB 5B1G6 and MAB 1F12E1 recognized the active form of maltase, found predominantly in the enzyme preparation from young animals but present, although less prevalent, in the enzyme isolated from aged animals. On the other hand, MAB 7G10H3 and MAB 2E1C10 were directed against an inactive form of maltase, found mainly in the enzyme preparation from senescent rats. This latter suggestion was supported by the experiment shown in Fig. 3 in which MAB 7G10H3 was used to estimate the relative amounts of the inactive enzyme moiety in various mixtures of maltase preparations from 6-mo- and 24-mo-old animals. When the mixture contained 0% old enzyme/100% young enzyme, the antibody bound was relatively small—only 15% of that bound when the mixture represented 100% old and 0% young enzyme preparations. As the percentage of old maltase in the fixed total protein mixture was increased, the amount of antibody bound increased, in a linear fashion.

Because of the findings that the MAbS would bind only to specific forms of maltase, immunoaffinity columns were developed with MAB 1F12E1 and MAB 7G10H3 as the ligands to separate the active from the inactive forms of the enzyme and to determine quantitatively the relative proportions of active and inactive enzyme in the maltase preparations from 6-mo- and 24-mo-old rats. Table 1 shows that 85% of the total maltase from 6-mo-old animals bound to MAB 1F12E1 and, thus, was in the active form of the enzyme. On the other hand, 15% of the total maltase from 6-mo-old rats bound to MAB 7G10H3 and, therefore, was in the inactive form. In contrast, with the maltase preparations from senescent animals, the proportionment of the enzyme bound to MAB 1F12E1 and MAB 7G10H3, respectively, indicated that only 41% of the total enzyme was in the active form, whereas 59% was inactive.

The maltase-IgG complexes eluted from the affinity columns were dissociated with NaDodSO₄ and 2-mercaptoethanol, and the subunit molecular weights were determined by gel electrophoresis. Fig. 4 shows that molecular sizes of the control preparations of maltase from young and aged animals and the active and inactive forms of the enzyme eluted from MAb

![Fig. 3. Estimation of the inactive form of maltase in a mixture of the enzyme from aged and young animals using the MAB synthesized by clone 7G10H3 as a probe. The amount of inactive enzyme in the mixture was determined by the ELISA assay.](image)

![Fig. 4. Autoradiograms of NaDodSO₄/polyacrylamide gels of ¹²⁵I-labeled maltase eluted from monoclonal IgG-protein A-Sepharose 4B affinity columns. The enzyme was processed as described in Table 1. The labeled eluents were lyophilized and incubated in 1% NaDodSO₄/1% 2-mercaptoethanol prior to electrophoresis on 3.75% polyacrylamide gel. Lanes: 1, young enzyme (control); 2, young enzyme eluted from MAB 1F12E1 affinity column; 3, old enzyme eluted from MAB 7G10H3 affinity column; and 4, old enzyme (control).](image)

### Table 1. Affinity binding of ¹²⁵I-labeled maltase to IgG-protein A-Sepharose 4B columns

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>MAB 1F12E1</th>
<th>MAB 7G10H3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maltase</strong></td>
<td>cpm</td>
<td>Enzyme, μg</td>
</tr>
<tr>
<td>Young*</td>
<td>2,850</td>
<td>2.83</td>
</tr>
<tr>
<td>(2,100–4,130)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old†</td>
<td>1,040</td>
<td>1.04</td>
</tr>
<tr>
<td>(910–1,240)</td>
<td></td>
<td></td>
</tr>
</tbody>
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*Average of three experiments.
†Average of four experiments.
The present study describes the development of four clones synthesizing antibodies to maltase, an enzyme localized in the renal proximal tubule brush border membrane. Because maltase is a well-established "marker" enzyme for the luminal segment of the proximal tubule cell plasma membrane (14), the potential application of this technique for the recognition and subsequent segregation of proximal tubule cells in a mixture of kidney cells is evident.

The findings in this paper demonstrate that the antigenicity of maltase is altered during the aging process. These observations indicate that young and old maltase have different epitopes and, thus, the data are in accord with and may reflect the age-dependent conformational change of the enzyme, previously reported (5). Of the four MAb's that recognize maltase, two (MAb 8B1G6 and MAb 1F12E1) bind maltase and immunoprecipitate enzyme activity. The other two (MAb 7G10H3 and MAb 2E1C10) also bind the enzyme but do not immunoprecipitate activity (i.e., all catalytic activity remains in the supernatant). MAb's synthesized by clones 7G10H3 and 2E1C10 react with a form of maltase found mostly in the aged rat. Indeed, about 60% of the maltase preparation from 24-mo-old animals is in this form. In contrast, only about 15% of the enzyme preparation from 6-mo-old animals is recognized by these antibodies. On the other hand, MAb's synthesized by clones 8B1G6 and 1F12E1 predominantly bind to the enzyme preparation from young rats, compared (on an equal protein basis) to the enzyme from the senescent animal. Of particular significance is the finding that the maltase which is recognized by MAb 7G10H3 and MAb 2E1C10 is enzymatically inactive. This increased prevalence of an inactive form of the enzyme in the aged rat can largely account for the decreased maltase-specific activity reported in the senescent animal (5). Moreover, the presence of inactive enzyme molecules negates the possibility that the difference in specific activity between the enzyme from young and old animals is due exclusively to a partial decrease in the catalytic activity of all maltase molecules. Instead, the decreased specific activity of the enzyme with age appears to be attributable to an increased proportion of inactive molecules relative to active molecules, although a combination of inactive and partially active enzymes has not been precluded.

Cleavage of the active and inactive enzymes with CNBr produces similar peptide maps, and dansylation of these peptides yields identical NH$_2$-terminal amino acids. This result suggests that the general chemical structures of the active and inactive maltase species are related, although a small alteration in composition has not been ruled out. However, the finding of an indistinguishable CNBr-peptide pattern does argue against the possibility that the inactive species is an unrelated contaminant. In addition, the inactive maltase, although devoid of catalytic activity, retains its Tris binding site, allowing the protein to be copurified with the active maltase (5). Because the chemical structures of young and old forms of maltase are similar, MAb's recognizing both forms are likely to be developed in future studies.

The development of antibodies specific to either young or old maltase species and their use as immun affinity ligands permits the separation of the active young maltase from the inactive aged form of the enzyme. This enables a determination of the relative proportions of active and inactive enzyme that are in the kidney of an animal of any age. Thus, a biological assessment of the aging process for the kidney, in addition to chronological age, may be envisioned. Moreover, the isolation of the two maltase species, one predominantly in young rats and the other mostly in the senescent animal, now permits the direct examination of critical questions on the mechanism of aging (e.g., are new gene products expressed during the aging process, and do molecular changes occur in preformed proteins, and, if so, what are the natures of these chemical alterations?). By utilizing separated active and inactive forms of the enzyme, these questions can be approached by a logical extension of the techniques and findings reported in this paper.

We wish to thank Ms. Lise Fluhr for technical assistance.