Immunoelectrophoretic Identification of Catalase Subcomponents in the Homogenates of Rat Tissues

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Summary

Immunoelectrophoretic analyses of catalase in a variety of tissues from normal Sprague-Dawley rats were made with rabbit anti-rat hepatic catalase serum. Several distinctive patterns of the enzyme were identified by this means. A pattern previously described for purified hepatic catalase with 6 immunologically independent subcomponents was again recognized in one of the chromatographically separated catalase fractions of the liver (RHC-A). Crude homogenates of the liver also were shown to contain the 6 subcomponents of the enzyme. Various combinations of the enzyme subcomponents were observed in different tissues, while the central nervous system and the ovaries revealed no catalatic activity or precipitin reaction by immunoelectrophoresis. These observations support the belief that catalase exists in multiple molecular forms and is, to some extent, characteristic of the tissue from which it is obtained.

Introduction

Immunoelectrophoretic evidence of subunits that may represent the fundamental constituents of catalase isozymes in human and rat tissues was recently reported from this laboratory (5). Beckman et al. (1) observed variants of catalase in maize endosperm and demonstrated that these enzymes were under the control of 2 codominant alleles. Their data revealed multiple electrophoretic bands of the hybrid enzymes, which exhibited mobilities that were intermediate between the parental catalases. Observations such as these suggest that catalase isozymes may assume several molecular forms in a variety of tissues, as has been shown for lactate dehydrogenase (3, 4).

Earlier data (5) indicated that hepatic catalase of the rat exists in at least 2 distinct forms, which have been designated RHC-A and RHC-B. The 2 fractions may be separated readily on Celite-diethylaminoethyl cellulose-calcium phosphate gel column and may be characterized by immunoelectrophoresis. The present communication reports upon the identification of multiple forms of catalase in crude rat tissue homogenates by immunoelectrophoresis.

Materials and Methods

Antiserum to purified hepatic catalase obtained earlier [5] was employed in the present study. The liver catalase used as the antigen has been described in detail (5); it possessed a substantially high specific activity of Kat f, 69,000. Catalase emulsified in complete Freund's adjuvant (Difco) was administered s.c. into young adult New Zealand rabbits for the induction of the antibody. One mg of catalase was injected every 3 days for 4 doses, and a final dose of 1 mg was given at the end of the 4th week. After an adequate titer was attained for immunodiffusion in agar (1:800/mg of antigen N), the sera were pooled. One control rabbit received an equivalent number of injections of Freund's adjuvant alone, and the serum from this rabbit was employed as "adjuvant control."

The tissues were obtained from freshly killed adult Sprague-Dawley rats of both sexes, weighing 200-225 gm. The organs listed in Table 1 were weighed (wet weights) after being rinsed in physiologic saline and prepared as 0.1-10.0% w/v homogenates in 0.1 M sodium phosphate buffer, pH 8.6, containing sufficient 1% Triton X-100 to constitute 0.1% of the final homogenate volume. A Potter-Elvejhem apparatus immersed in an ice bath was used for the homogenization. The final concentration of each homogenate preparation was determined by the relative activity of catalase, which was estimated through assays (see Table 1).

The method of assay for catalase in tissues by titrimetric procedure was described previously (5), and the nitrogen content of the homogenates was determined by micro-Kjeldahl procedure. The catalase activities of the homogenates are expressed as the 1st order constant, K, at time zero, which was obtained by extrapolation through the points at 15, 30, 45, and 60 sec (7).

The values are reported here as the reaction constant per mg of tissue nitrogen, or K/N.

The procedure employed for immunoelectrophoresis of catalase was detailed elsewhere (5) and is presented briefly: Ionagar

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2 Career Development Awardee USPHS No. 2-K3-GM-244-06.
3 The following abbreviations are used: RHC-A, 1st chromatographic fraction or fraction A of rat hepatic catalase (5); RHC-B, 2nd chromatographic fraction or fraction B of rat hepatic catalase (5); Kat f, Katalsfäigkeit; K, 1st order reaction constant; PHC, purified rat hepatic catalase; DEAE, diethylaminoethyl cellulose.
4 For definition, see Ref. 14.
TABLE 1
CONCENTRATIONS OF TISSUE HOMOGENATES USED FOR IMMUNOELECTROPHORESIS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentration [% wet wt. (w/v)]</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>1.0</td>
<td>Concentrated to 0.1 original hemolysate volume</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.0</td>
<td>Slight enzyme activity &gt; 10%</td>
</tr>
<tr>
<td>Small intestine</td>
<td>1.0</td>
<td>Slight enzyme activity &gt; 10%</td>
</tr>
<tr>
<td>Testis</td>
<td>2.0</td>
<td>No activity at 50% concentration</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>10.0 or greater</td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>10.0 or greater</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>10.0 or greater</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2
Tissue | CATALASE ACTIVITY (K/N)* | IMMUNOELECTROPHORETIC COMPONENTS USING HEPATIC CATALASE ANTISERUM | TOTAL NO. OF COMPONENTS |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.24</td>
<td>1                  2                  3                  4                  5                  6</td>
<td>6</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.37</td>
<td>0.29               0.33               +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  6</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.04</td>
<td>0.06               0.05               -                  +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  4</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.03</td>
<td>0.05               0.04               +                  +                  +                  +                  +                  -                  +                  +                  +                  +                  +                  4</td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>0.04</td>
<td>0.04               0.04               +                  -                  +                  +                  +                  +                  +                  +                  +                  +                  +                  4</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.03</td>
<td>0.04               0.035               -                  +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  3</td>
<td></td>
</tr>
<tr>
<td>Sm. Intestine</td>
<td>0.03</td>
<td>0.03               0.03               -                  +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  4</td>
<td></td>
</tr>
<tr>
<td>Skel. Mus.</td>
<td>0.01</td>
<td>0.02               0.015             -                  +                  +                  +                  +                  +                  +                  +                  +                  +                  +                  2</td>
<td></td>
</tr>
<tr>
<td>Brain and Sp. Cord</td>
<td></td>
<td>0                  0                  0                  -                  -                  -                  -                  -                  -                  -                  -                  -                  -                  0</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td></td>
<td>0                  0                  0                  -                  -                  -                  -                  -                  -                  -                  -                  -                  -                  -                  0</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>0.37</td>
<td>0.29               0.33               -                  -                  -                  -                  -                  +                  +                  +                  +                  +                  +                  1*</td>
<td></td>
</tr>
</tbody>
</table>

* K/N, the 1st order constant (reaction constant) per mg of tissue nitrogen.
* Mean value of organs from 3 rats.
* Erythrocyte catalase has 6 components (see Fig. 4), but only 1 of these reacts with anti-rat hepatic catalase (see text).

No. 2 (Oxoid Ltd., London) prepared as 1.15% solution containing calcium lactate and Veronal buffer in the manner described by Hirschfeld (2), was layered uniformly (2-mm thickness) on glass slides, 50 x 75 mm. Troughs and wells were prepared in the standard manner, and 0.05-0.1 ml of homogenate samples were delivered into each of the antigen wells. Electrophoresis was conducted in the cold room at 5°C, and about 23 volts/cm of slide width were maintained with constant current for a duration of 2 hr. Precipitin reaction was developed by placing 0.25 ml of rabbit anti-rat hepatic catalase serum into each of the troughs (except in instances where tests were performed with rabbit anti-rat erythrocyte catalase serum) and allowing the reaction to proceed from 24–48 hr at 5°C in a moist chamber. Control serum was used for all homogenates examined by agar gel immunoelectrophoresis.

Immunoelectrophoretic patterns of highly purified rat hepatic catalase isozymes, designated RHC-A and RHC-B (5), were determined, and comparisons were made with those observed for a less pure catalase preparation of rat livers, as well as with those observed for the crude liver homogenates. The identities of all subcomponents in the immunoelectrophoretic patterns of the crude tissue homogenates were established by such comparisons.

Purified rat erythrocyte catalase, Kat f 120,700 (5), and the specific antiserum prepared against it as reported previously (5) were used to characterize the crude erythrocyte catalase pattern. Rat erythrocytes were hemolyzed with an equal volume of distilled water after the cells had been washed 3 times in physio-
logic saline solution. The hemolysate was then assayed for catalase activity and nitrogen content. An aliquot of the hemolysate was placed in a dialysis tube and concentrated to 0.1 of the initial volume by exposure to a rapidly moving mass of cold air. The concentrated hemolysate was again assayed and was finally used for immunoelectrophoretic analysis.

**Results**

Significant variations in catalytic activity were observed for different tissue homogenate samples. When activities, expressed as $K/N$, are arranged in a receding order (Table 2), it is evident that the liver constitutes the richest source of catalase. Erythrocytes and kidney give intermediate values, and other tissues are generally much lower; the central nervous system\(^5\) and ovary seem to have no activity within the range of homogenate concentrations tested in the present study.

\(^5\) Brain and spinal cord at concentrations much higher than 10% exhibited a weak catalytic reaction.

Immunoelectrophoretic patterns of the purified enzyme were highly reproducible, as before. Two catalase fractions separated from the purified liver catalase (PHC) on DEAE-Celite-calcium phosphate gel column were again identified by immunoelectrophoresis. Fraction RHC-A is seen to consist of 6 arcs (Figs. 1 and 2A), while RHC-B has been shown to contain only 4.\(^6\) PHC (Fig. 1) characteristically yields a 6-arc pattern similar to those of RHC-A, and this is to be expected since fraction RHC-B contains 4 arcs that are identical with arcs 2, 3, 4, and 5 of RHC-A. Immunoelectrophoresis of crude liver homogenates also yields 6 arcs, but as seen in Fig. 2C, the rates of diffusion through the gel medium of some of the components differ from those observed in RHC-A. This variation in diffusion is present to a lesser degree in the partly purified hepatic catalase (Fig. 2B). Hence, it appears that the diffusion of certain of the enzyme components through agar gel may be modified after

\(^6\) Preliminary observations registered 4 arcs for fraction RHC-B (5), but recent studies indicate that the correct number may be 3 (unpublished observation).
Immunoelectrophoretic Analysis of Catalase

VARIATIONS IN IMMUNOELECTROPHORETIC ARCS OF TISSUES

FIG. 4. Immunoelectrophoretic patterns of: A, purified erythrocyte catalase; B, concentrated hemolysate of erythrocytes.

FIG. 5. Immunoelectrophoretic patterns of crude liver homogenate reacted against anti-rat hepatic catalase serum (patterns at left of each illustration) and against anti-rat erythrocyte catalase serum (patterns at right of each illustration).

CHART 1. Graphic summary showing the distributions of catalase subcomponents in various tissues.

chromatographic purification as noted in fraction RHC-A. The comparison of patterns between RHC-A and crude liver homogenates allows for a reasonably precise identification of the 6 subcomponents, as shown in Fig. 2.

A striking variation in the patterns of catalase subcomponents is seen when immunoelectrophoresis of crude tissue homogenates is examined with rabbit anti-rat hepatic catalase serum. These patterns are illustrated in Fig. 3 with photographs and accompanying line drawings. The pattern that is characteristic for kidney homogenate was identical with that observed for the liver and is not shown in the illustration. The spectrum of patterns ranged from the complex configuration of liver tissue to a relatively simple configuration that consisted of only 2 arcs for the skeletal muscle. Brain, spinal cord, and ovary yielded no precipitin reaction, nor was any enzymatic activity detected in these tissues.

The crude hemolyzed samples of erythrocytes examined by immunoelectrophoresis using rabbit anti-rat erythrocyte catalase serum disclosed a 6-arc pattern that was identical with the pattern described for purified rat erythrocyte catalase (5). As in the case of purified erythrocyte catalase, the arcs were seen to migrate in pairs. A total of 3 paired arcs was observed, as shown in Fig. 4.

The antigenic relationships among the various subcomponents of the purified liver catalase, fraction RHC-A, and the subcomponents of purified erythrocyte catalase were examined. Electrophoresis of RHC-A was carried out in Ionagar No. 2 as described above, and the fraction was allowed to react with its homologous serum, rabbit anti-rat hepatic catalase serum, in one trough and with rabbit anti-rat erythrocyte catalase serum in an adjacent trough. The result seen in Fig. 5 shows that RHC-A and rat erythrocyte catalase, each of which has 6 components, have only 1 component with a common antigenic relationship. The common arc is tentatively identified as arc No. 5 of RHC-A by its position in the immunoelectrophoretic pattern. The result observed here (Fig. 5) shows that liver and erythrocyte catalases are isozymes—but that 1 of the subcomponents is possessed in common by both.

Crude tissue homogenates of several organs examined in this fashion by the utilization of rabbit anti-rat erythrocyte catalase serum disclosed little additional information, since all tissues were apparently insufficiently free of erythrocytes and yielded variable arc patterns. The patterns that characterized various tissues were seen only when the tissues were tested with rabbit anti-rat hepatic catalase serum; they are summarized in Chart 1.

Discussion

As early as 1938, Sumner and Gräfen (12) demonstrated that native bovine hepatic catalase, which has a sedimentation coefficient of 11.3 S, could be dissociated into smaller, denatured components by drastic alteration of pH in either highly acidic or alkaline ranges (pH 2.8 and pH 9.9). More recently, Tanford and Lovrien (13), in their analysis of commercial bovine catalase, noted a fraction with high specific activity and another of low value. With the high activity fraction, the authors found sedimentation peaks at 7.2 S and 4.5 S when the pH was altered, in addition to the native catalase peak of 11.6 S. They attributed the 7.2 S and 4.5 S peaks to $\frac{1}{2}$-size and $\frac{1}{2}$-size molecular units. Samejima and co-workers (10, 11), employing the method of altering pH as well as by denaturation with formamide and urea, were able to dissociate bovine liver catalase into smaller sub-
components and demonstrated that these, in turn, were capable of renaturation with the return of enzymatic activity. Similar observations have been reported by Saha et al. (9). Samejima and Shibata (10) concluded that liver catalase of bovine origin in all probability consists of either 4 or 6 subunits. The data presented by Tanford and Lovrien (13) are, however, more consistent with the thesis of 4 subunits/molecule. Currently, a belief prevails that catalase is a hemoprotein consisting of 4 hemin groups/molecule. If catalase proves to be a tetramer as suggested by Tanford and Lovrien, this view would conform well with the 4 iron porphyrin moieties proposed for the molecule. The 4-porphyrin concept, however, does not militate against the view that there may be 6 subunits, as suggested by Samejima and Shibata (10) for bovine catalase and by Nishimura et al. (5) for rat hepatic and erythrocyte catalases. No analytic data are as yet available for the iron determinations of such proposed polypeptide subunits.

Upon comparison of catalase subcomponents of various tissue homogenates with the results reported for isozymes of lactate dehydrogenase (3, 4), the distribution of catalase subcomponents is seen to be reminiscent of that reported for the latter enzyme. Detectable subcomponents of catalase vary from 0 to 6 in the tissues examined (Table 2). It is to be stressed that the patterns observed were obtained specifically with rabbit antiserum prepared against rat liver catalase, and the data must be interpreted within this framework. Although some of the tissues exhibited a deficiency in certain of the subcomponents, it is entirely possible that there are other subcomponents, like those in erythrocyte catalase, that are antigenically unrelated to those normally encountered in the liver. One body of evidence against this view, however, is the reasonably good correlation observed between the enzymatic activity of tissues and the numbers of subcomponents observed in the corresponding tissues. This relationship is tabulated in Table 2. It may be noted that liver and kidney have 6 subcomponents and represent tissues of highest catalytic activity. At the opposite end of the scale are noted skeletal muscle, 2 components of which have minimal enzymatic activity, and brain, spinal cord, and ovary, which showed a total deficiency in catalase activity. At the opposite end of the scale are noted skeletal muscle, 2 components of which have minimal enzymatic activity, and brain, spinal cord, and ovary, which showed a total deficiency in catalase activity. Small intestinal mucosa was the only exception in that as many as 4 arcs were observed despite a very low catalytic activity. Tissues of the central nervous system in our rat material appeared to lack catalase, but it is noteworthy that positive staining for catalase was observed in the myelin and ganglion cells of mouse spinal cord (Nishimura, E. T., unpublished observation) by a fluorescent antibody technic (6). A qualitative enzymatic test for catalase, moreover, was positive for gray and white matter of human brain, especially after the tissues were defatted with 95% ethanol (E. T. Nishimura, unpublished observation). Such data are seemingly contrary to those observed for the rat brain and spinal cord. There is no reasonable explanation for this discrepancy, but the cause, in some manner, may be related to the lipid-rich tissues in which the enzyme is bound as, e.g., in myelin. Such an arrangement may perhaps interfere with the reactive sites of the enzyme molecule with respect to both substrate and antibody in an artificial system of testing such as described here.

The disturbance and alteration of catalase synthesis in neoplastic tissues has been well documented in recent years (8). The identification of catalase subcomponents by immunoelectrophoresis may serve as an important adjunct in the study of the altered biosynthesis of this enzyme by the neoplastic cells. The problem of repressed synthesis of liver catalase during extrahepatic tumor growth (7) also may be clarified through a more precise knowledge of the enzyme subcomponents. These and other related problems are currently being investigated.

References

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