Homospecific Activity, an Immunologic Index of Enzyme Homogeneity; Changes During the Purification of Dopamine-β-Hydroxylase

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ABSTRACT

The ratio of enzymatic activity to homologous enzyme protein (measured immunologically) can be used as a sensitive index of enzyme homogeneity. The name *homospecific activity* is proposed for this parameter (units of enzyme activity per mg of antigen). Measurements of *homospecific activity* allowed detection of inactive forms of dopamine-β-hydroxylase which accumulated during some purification procedures and which were not separable from active enzyme by standard analytical procedures. *Homospecific activity* is proposed as a routine index of homogeneity during enzyme purification.

INTRODUCTION

It is generally assumed that the specific activity of an enzyme preparation (i.e., activity per unit of protein) attained when the preparation appears homogeneous by accepted analytical procedures, is an adequate criterion for purity. However, the reported specific activities for apparently homogeneous preparations of dopamine-β-hydroxylase (DβH) vary as much as ten fold (1-6). Since this variation appears to be too great to be the result of contamination by other proteins, we have sought to determine whether it may be due to the presence of an enzymatically inert form of DβH that co-purifies with the active form and is not separable from it by standard analytical procedures. To do so,

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we have utilized a specific radioimmunoassay to measure DβH protein during purification. The ratio of enzyme activity to homologous enzyme protein yields a new parameter of specific activity (units of enzyme activity per mg of antigen) which can be measured precisely even in the crudest preparations and for which we have previously suggested the term *homospecific activity* (7). This report describes the use of this parameter for the analysis of DβH in tissue extracts and during purification.

METHODS

The catalytic activity of DβH was determined using the photometric assay described by Nagatsu and Udenfriend (8). The specific activity of the enzyme is expressed in μmoles of octopamine formed per minute per mg of protein. The amount of DβH protein present was estimated using the solid phase radioimmunoassay described elsewhere (9). DβH purified by affinity chromatography (5) was used as a standard. The ratio of enzyme activity to total enzyme protein is expressed in μmoles of octopamine formed per minute per mg of DβH protein, and is referred to as *homospecific activity*. Protein was determined by the method of Lowry et al. (10) using bovine serum albumin as a standard. The purity of each enzyme preparation was verified using polyacrylamide disc gel electrophoresis according to the method described by Davis (11).

Purification of DβH

All procedures were carried out at 2°C. DβH was purified both by the original multistep procedure of Friedman and Kaufman (1), as well as by a more gentle and rapid affinity chromatography technique (5).

Method (a) For the former procedure, sixty bovine adrenals were obtained fresh from the slaughterhouse, transported on ice and used immediately. The medullae were removed, homogenized in 5 volumes of 10 mM sodium acetate buffer pH 6.0, using a Polytron homogenizer (Kinematica, Switzerland) for 60 secs at a setting of 5. After centrifugation at 700 x g for 10 min, the supernatant was decanted through two layers of cheese-cloth to remove lipids, and recentrifuged at 26,000 x g for one hour. This supernatant fraction contained sufficient of the soluble form of the enzyme and was therefore used for the purification. To each 100 ml of this solution, 55 g of ammonium sulfate were added. The precipitate was collected by filtration on Whatman No. 54 filter paper, dissolved in 0.02 M buffer and dialyzed overnight. At this stage an ammonium sulfate fractionation was performed as described by Friedman and Kaufman (1) and the enzyme was purified further according to the procedure set out in that report.

Method (b) Purification by affinity chromatography on a column of concanavalin A-Sepharose was carried out as described in an earlier publication (5), except that catalase (50 μg/ml) was added to both the 0.3 M and 1.6 M sucrose solutions.

RESULTS

Purification of DβH

Both preparations of DβH (Methods a and b) were shown to be homogeneous by polyacrylamide disc gel electrophoresis. Table I(a) summarizes changes

TABLE I

(a) INACTIVATION OF DβH DURING PURIFICATION

Purification Procedure or Fraction	Specific Activity (Units/mg protein)	Homospecific Activity (Units/mg DβH)
$(NH_4)_2SO_4$:(80%)	0.043	23.9
$(NH_4)_2SO_4$:(14.7% - 21.7%)	0.098	17.6
Calcium Phosphate Gel	0.55	16.3
Ethanol	0.55	12.3
DEAE-Cellulose	1.27	11.9
Sephadex G-200	13.20	12.9

(b) PURIFICATION OF DβH USING CONCANAVALIN A

Chromaffin Vesicle Lysate	0.29	20.0
Eluate from Concanavalin A column	18.9	20.3

I(a) Dopamine-β-hydroxylase was purified by the procedure shown and the specific activity and homospecific activity determined at each step.

I(b) The soluble lysate from isolated bovine chromaffin vesicles was applied to a column of concanavalin A-Sepharose. After extensive washing, elution was performed with 10% α-methyl-D-mannoside in 50 mM potassium phosphate buffer, pH 6.5.

observed in the specific activity and homospecific activity at each step in the isolation of DβH by the Friedman and Kaufman technique (Method a). It can be seen that although the specific activity of the enzyme increased at each step in the isolation, to yield a 307 fold purification, the homospecific activity actually decreased by approximately two fold, from 23.9 to 12.9 units per mg DβH. Thus, not only did inactivation of the enzyme occur during the isolation procedure, but in addition, the inactive form(s) of the enzyme failed to separate from the active form by all the procedures employed. The amount of DβH protein

that could be accounted for after each step in the isolation was always greater than 87%, whereas recovery of DβH activity was always lower than 85%, and at one step was only 65%.

Isolation of DβH by the more rapid and gentle procedure of affinity chromatography on a column of concanavalin A-Sepharose (Method b) allowed the preparation of purified DβH with the same homospecific activity as that of the starting material, and hence without inactivation. As can be seen from Table I(b), the specific activity of the final preparation (18.9 units) was much higher than that isolated by the previous procedure.

Comparison of Active and Inactive Forms of DβH: Purified soluble DβH was heated at 60°C for 60 mins, and assayed for both enzymatic and immunological activity. The enzymatic activity of the treated enzyme was completely lost, whereas no loss of immunological activity could be detected. When the active and the heat inactivated forms of DβH were subjected to polyacrylamide disc gel electrophoresis, no difference in the electrophoretic mobility of the two forms could be observed. In addition, the inactivated enzyme was also bound by concanavalin A, indicating that no dramatic alteration had occurred to the carbohydrate moiety.

When chromaffin vesicles were prepared without the addition of catalase to all solutions used during purification, the soluble DβH obtained after lysis was found to be unstable. Enzymatic activity was lost completely after storage on ice for only 24 hours. However, there was no loss of immunological reactivity at this time, and the protein was still bound by concanavalin A. Thus it was possible to "purify" the completely inactive enzyme by the procedure described in Method (b). Both the specific activity and the homospecific activity of this preparation were of course zero. Storage of this preparation for shorter periods led to intermediate values for homospecific activity.

Homospecific Activity of DβH in Crude Extracts: Eleven adrenal medullae were individually homogenized in a Polytron homogenizer and centrifuged at 20,000 x g for 30 mins. The homospecific activity of DβH in each of the supernatants did not vary greatly (Av. 18.8 units per mg DβH; range 15.9 - 22.5).

TABLE II

HOMOSPECIFIC ACTIVITY OF SOLUBLE AND MEMBRANE-BOUND DβH

Gland	Soluble DβH (units/mg DβH)	Membrane-Bound DβH (units/mg DβH)
1	18.5	4.6
2	20.5	3.5
3	21.1	4.7
4	23.2	4.9
5	20.4	3.8
Mean	20.7	4.3

The homospecific activity of dopamine-β-hydroxylase was determined in both the soluble and particulate fractions of chromaffin vesicles isolated from individual bovine adrenal glands.

When chromaffin vesicles were isolated from individual adrenal glands (Table II) the homospecific activity of the soluble enzyme (Av. 20.7 units per mg DβH; range 18.5 - 21.1, n = 5) was found to be almost 5 fold greater than for the membrane bound enzyme solubilized with Triton X-100 (Av. 4.3 units per mg DβH; range 3.5 - 4.9, n = 5). In order to determine whether the Triton X-100 used in the solubilization procedure caused some interference in the radioimmunoassay, two control experiments were performed. Firstly, the recovery of purified DβH added to the samples was found to be 120%. Secondly, when the soluble DβH was treated with Triton X-100, in a manner identical to the membrane bound DβH solubiliztion procedure, the value obtained was only slightly higher than the original measurement (108%). The detergent does not inhibit enzyme activity. Thus the lower homospecific activity of the membrane-bound enzyme cannot be explained by Triton X-100 interference.

DISCUSSION

The use of radioimmunoassays for the determination of an ever increasing

number of antigens has provided many disciplines with a powerful new tool since its introduction in 1960 (12). However, its use in enzymology has been often overlooked and the first report of a radioimmunoassay for an enzyme did not appear until as late as 1969 (13), despite the obvious advantages of increased sensitivity and independence from cofactors and inhibitors.

The results presented in this paper emphasized another important attribute of enzyme radioimmunoassay. When combined with a catalytic assay it then becomes possible to detect catalytically inert forms of an enzyme. The loss of enzyme activity due to inactivation, throughout a lengthy purification procedure, could perhaps be expected. However, the fact that the inactivated enzyme fails to separate from the native DβH, as shown by the decrease in homospecific activity, is not so readily predictable. We have previously reported a specific activity of 8.7 units per mg protein for DβH purified on concanavalin A (5). That preparation was isolated without the addition of catalase and it is therefore likely that it also contained inactive enzyme. The specific activity of the bovine DβH purified by affinity chromatography during these experiments (18.9), is the highest reported so far. The fact that no inactivation of the enzyme occurred during the isolation suggests that this more closely resembles its native state. Further, since the homospecific activity of the supernatant fractions showed only small variation from gland to gland, it can be concluded that either the amount of inactive enzyme present "in vivo" is constant from animal to animal, or that there is no inactive enzyme present and little inactivation during homogenization.

The lower homospecific activity of the membrane-bound DβH may represent a true difference in this form of the enzyme from the soluble form. It indicates that membrane extracts contain far more enzyme protein than is apparent from their enzyme activity. Such information can only be obtained by an immunological methods. Hortnagl et al. used a micro complement fixation procedure (14) to show immunological differences between membrane-bound and soluble DβH comparable to those found here.

It is likely that inactivation such as that reported here for DβH also occurs

during the purification of other enzymes. Changes in homospecific activity may explain the more than 4 fold difference in specific activity reported for two preparations of chick embryo prolyl hydroxylase obtained by different purification procedures (15, 16); both preparations were "homogenous" by disc gel electrophoresis. As in the case of DβH the prolyl hydroxylase preparation with the higher activity was obtained by a rapid affinity chromatography procedure (17). Immunologic methods have been used from time to time to detect inactive forms of enzymes, in cases where they were suspected. They have not been employed as a routine part of enzymology. We suggest that homospecific activity be introduced as a standard criterion of homogeneity during enzyme purification.

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