BOVINE ADRENAL MEDULLA TYROSINE HYDROXYLASE: SEPARATION OF THE NATIVE AND AGGREGATE FORMS

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1. Introduction

Tyrosine hydroxylase catalyzes the rate-limiting step in the biosynthesis of catecholamines [1]. In spite of the interest of this enzyme, especially in view of its important regulatory role, the bovine adrenal medulla tyrosine hydroxylase has not yet been purified in its native form.

Homogenization in isotonic sucrose yields a 155 000 dalton enzyme and a higher mol. wt form, the particle-bound enzyme [2,3]. Aggregation can be avoided by homogenization in buffer containing isotonic salt but is induced by subsequent fractionation using ammonium sulfate [4]. Therefore aggregation is an artefact occuring during the preparation and is an obstacle to the purification of the native enzyme [3].

To avoid aggregation, purifications of the adrenal enzyme have been carried out on a 'solubilized' enzyme obtained by proteolytic digestion of the particle-bound form [5–7]. The trypsin-solubilized enzyme is mol. wt 34 000. After solubilization, tyrosine hydroxylase no longer aggregates.

Studies of adrenal tyrosine hydroxylase have been performed with the proteolyzed active fragment of the enzyme, or with the particulate enzyme, or with the ammonium sulfate precipitate which contains both the native and aggregate forms, but never with the native enzyme alone.

In this paper, the high mol. wt form has been eliminated from the enzyme preparation and the evidence obtained indicates that there is no equilibrium between the two forms of the enzyme. Such an observation will allow further purification of the native adrenal tyrosine hydroxylase.

2. Materials and methods

2.1. *Materials*

L-[3,5-^{3}H]Tyr, 40 mCi/mmol, was obtained from the Radiochemical Center (Amersham, Bucks). It was further purified on Dowex X-50 before use [8]. The artificial cofactor 6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) was purchased from Aldrich. Dithiothreitol (DTT) was obtained from Sigma.

2.2. *Standard enzyme assays*

For assay of tyrosine hydroxylase activity, incubation mixtures contained Tris–acetate buffer 10^{-1} M, pH 6.2, 2-mercaptoethanol 2×10^{-2} M, $DMPH_4$ 5×10^{-4} M (in HCl 5×10^{-3} M bubbled under argon before use), L-[3,5-^{3}H]Tyr 5×10^{-4} M (450 000 cpm) and tyrosine hydroxylase in final vol. 1 ml. Incubation was for 15 min at 37°C. The reaction was stopped by addition of 50 µl glacial acetic acid. The tritiated water produced was assayed by the method in [9]. Blank values obtained by omitting the enzyme and adding the same volume of buffer were subtracted from the radioactive measurements of ^3H_2O.

Activity of yeast alcohol dehydrogenase was determined directly by measuring spectrophotometrically the initial rate of reduction of NAD by ΔA_{340}.

2.3. *Preparation of tyrosine hydroxylase*

All procedures were performed at 3°C. Fresh bovine adrenal medullae (30 g) were minced, homogenized in 2 vol. KCl 1.19% (isotonic) in buffer Tris–HCl 5×10^{-3} M, final pH 7. Homogenization was carried out in a Virtis blender operated at 17 000 rev/min for 1 min. After centrifugation at 37 000 × *g* for

90 min, solid ammonium sulfate (140 g/litre) was added to the supernatant. The centrifuged precipitate was discarded and solid ammonium sulfate (140 g/litre) was added to the supernatant. During precipitation the pH was kept at 7. After centrifugation, the pellet was dissolved in 5×10^{-2} M Tris–HCl, pH 7.5, containing 2×10^{-3} M DTT (this buffer was always used thereafter). The extract was then purified on Sepharose 6B columns as described in fig.1.

2.4. *Sucrose gradient centrifugations*

Linear sucrose gradients (5–20%) were prepared in buffer. The samples of tyrosine hydroxylase mixed with yeast alcohol dehydrogenase (150 μg) were layered onto 5.1 ml gradients. The gradient tubes were centrifuged in a SW 65 rotor for 14 h at 40 000 rev/min at 3°C in a Beckman L 2-65 B centrifuge. After centrifugation, the bottom of tubes were pierced and fractions (154 μl) collected and assayed for tyrosine hydroxylase activity. The pellets were resuspended in 150 μl buffer and assayed under the same conditions.

3. Results and discussion

3.1. *Isolation of two forms of the enzyme*

When tyrosine hydroxylase preparations obtained by ammonium sulfate fractionation were chromatographed on Sepharose 6B columns, the elution profile showed two active peaks (fig.1A). Peak I was eluted in the void volume and thus corresponded to the high mol. wt species (higher than 4×10^6 according to Pharmacia standards). The other active fractions (peak II) were retained by the gel Sepharose 6B. Peaks I and II are assumed to contain the aggregate and the native form of the enzyme, respectively.

A similar separation was obtained by centrifugation on a sucrose gradient (fig.2). The same tyrosine hydroxylase preparation was used and dialyzed against buffer before being centrifuged. The sedimentation pattern given in fig.2A confirms the results obtained under similar conditions [4] showing a peak of native enzyme, an aggregate at the bottom of the tube and an appreciable amount of intermediate molecular weight species in the lower portions of the gradient.

It can be observed that peaks I and II (fig.1A) amounted to 38% and 49%, respectively, of the total activity recovered after the Sepharose column and that the pellet after centrifugation on a sucrose gradient contained 43% total recovered activity. The

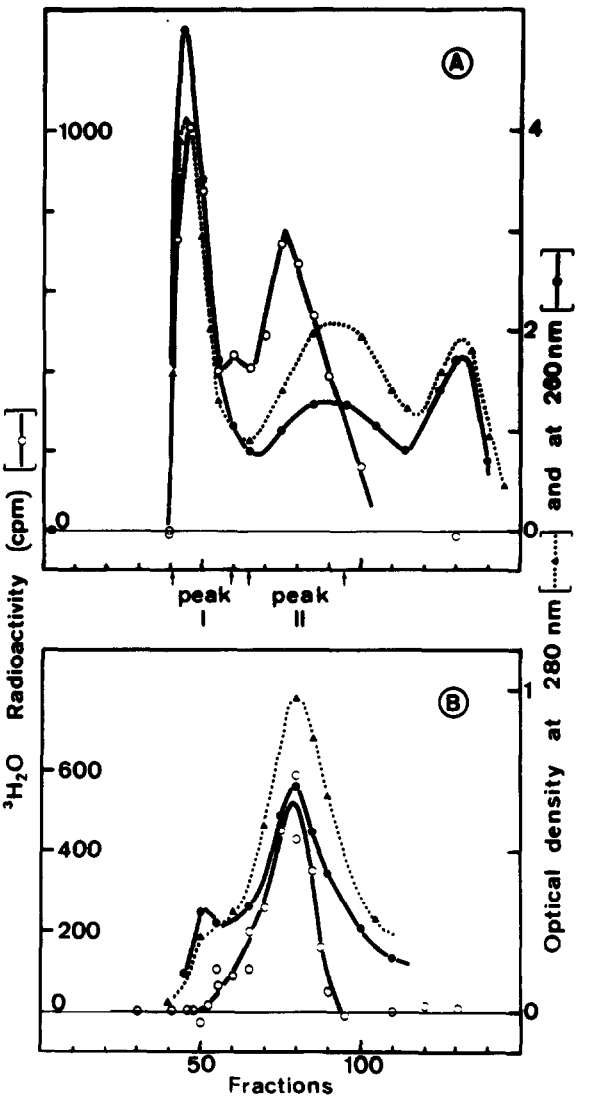

Fig.1. Elution pattern of tyrosine hydroxylase on a Sepharose 6B column (4 × 44 cm) in 5×10^{-2} M Tris–HCl, pH 7.5, DTT 2×10^{-3} M buffer. Fraction volume was 3.8 ml. (A) The sample applied was an ammonium sulfate precipitate prepared as described in section 2. (B) The sample applied came from the pooled fractions of peak II from the first column (see fig.1A) precipitated with ammonium sulfate (80% saturation).

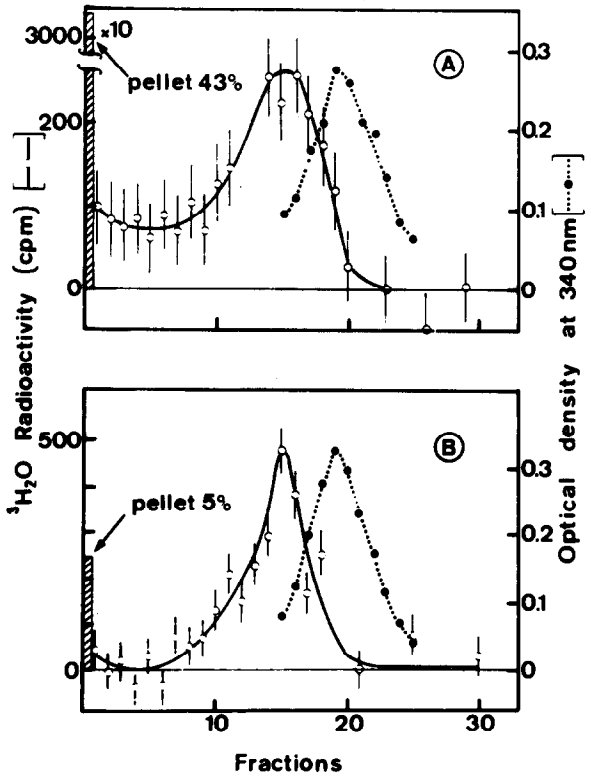

Fig.2. Sedimentation pattern of tyrosine hydroxylase centrifuged on sucrose gradients. (A) Tyrosine hydroxylase was prepared by ammonium sulfate precipitation as described in section 2 and layered onto a sucrose gradient (5–20%) with alcohol dehydrogenase. (B) Tyrosine hydroxylase was the pooled fractions of peak II from the first column of Sepharose (see fig.1A) precipited with ammonium sulfate (80% saturation) then dialyzed against buffer and centrifuged on a linear sucrose gradient. The precision $\sqrt{2N}$ of each value N of ^3H_2O activity measured (cpm) is given on the figure.

similar figures obtained by the two techniques for the ratios of the two forms further suggest that these two separations are equivalent and that peak II contains the native enzyme.

3.2. *Does an equilibrium exist between the two forms?*

Since under our conditions of preparation the aggregated form of tyrosine hydroxylase was not negligible, Sepharose 6B columns might be helpful in preparing the native enzyme. Nevertheless before using it as a purification step it had to be shown that after Sepharose 6B chromatography the enzyme no longer aggregated. Aggregation would again occur if the two forms were in equilibrium. Another possibility is that aggregation is mediated by some endogenous molecules liberated during the homogenization procedure and is further favored by high concentration or precipitation by ammonium sulfate.

Two types of experiment have been done with the isolated native enzyme to assess these hypotheses. Peak II (fig.1A) was precipitated with ammonium sulfate at 80% saturation and the pellet was resuspended in buffer and used for both experiments. A second gel filtration of this sample was carried out some hours after the first one and under exactly the same conditions. The elution diagram (fig.1B) showed nearly all the tyrosine hydroxylase activity in the peak of native enzyme. A very small amount of enzyme activity was eluted in the void volume indicating a very slight contamination by the aggregate in the pooled fractions of peak II. Thus, after separation from the aggregate and from a putative endogenous compound, the native enzyme no longer aggregated, even after a further precipitation with ammonium sulfate. Moreover, there is no effect of enzyme concentration on aggregation. A good confirmation of this was obtained by centrifugation on a sucrose gradient of the dialyzed native enzyme (peak II). Whereas 95% total activity recovered was found in the peak, only 5% was in the pellet (fig.2B). During centrifugation, sucrose did not induce formation of the high mol. wt species as it did during the homogenization procedure.

Although the composition of the aggregate is unknown, it should be noted that it is found in fractions containing large amounts of nucleic acids as shown by the high A_{260} values. The same observation has been reported [10,11]; the aggregates found during purification of brain tyrosine hydroxylase [10] and of tryptophan hydroxylase [11] contained nucleic acids and in the last case the aggregate must be removed from the preparation to avoid unspecific adsorption of the enzyme during purification. That nucleic acids stick to the hydroxylase is not unlikely since it is known that polyanions such as polyglutamate, phospholipid, or heparin interact with the brain enzyme [12,13]. This type of interaction might be important in vivo, since it results in a change of the enzyme properties and thus may have regulatory functions.

Acknowledgements

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References

[1] Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965) J. Pharmacol. Exp. Ther. 148, 1–8.
[2] Laduron, P. and Belpaire, F. (1968) Nature 217, 1155–1156.
[3] Wurzburger, R. J. and Musacchio, J. M. (1971) J. Pharmacol. Exp. Ther. 148, 1–8.
[4] Musacchio, J. M., Wurzburger, R. J. and D'Angelo, G. (1971) Mol. Pharmacol. 7, 136–146.
[5] Petrack, B., Sheppy, F. and Fetzer, V. (1968) J. Biol. Chem. 243, 743–748.
[6] Shiman, R., Akino, M. and Kaufman, S. (1971) J. Biol. Chem. 246, 1330–1340.
[7] Hoeldtke, R. and Kaufman, S. (1977) J. Biol. Chem. 252, 3160–3169.
[8] Ikeda, M., Fahien, L. A. and Udenfriend, S. (1966) J. Biol. Chem. 19, 4452–4456.
[9] Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) Anal. Biochem. 9, 122–126.
[10] Joh, T. H. and Reis, D. J. (1975) Brain Res. 85, 146–151.
[11] Joh, T. H., Shikimi, T., Pickel, V. M. and Reis, D. J. (1975) Proc. Natl. Acad. Sci. USA 72, 3575–3579.
[12] Katz, I. R., Yamauchi, T. and Kaufman, S. (1976) Biochim. Biophys. Acta 429, 84–95.
[13] Kuczenski, R. T. and Mandell, A. J. (1972) J. Biol. Chem. 247, 3114–3122.