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pH-dependent release of catecholamines from tyrosine hydroxylase and the effect of phosphorylation of Ser-40

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Bovine adrenal tyrosine hydroxylase (TH) is isolated in a partially inhibited state with the feed-back inhibitors adrenaline and noradrenaline tightly coordinated to high-spin (S = 5/2) Fe(III) at the active site. In addition to the charge-transfer interaction with iron, an additional charged group in the polypeptide chain, with an apparent pK_a of about 5.3 at 4°C, is involved in the binding of catecholamines. Protonation of this group increases the pseudo-first order rate constant for the dissociation of the TH-[^{3}H]noradrenaline complex more than 100-fold at 4°C. At pH 7.0 and 30°C, phosphorylation of Ser-40 causes a 6-fold increase in the rate constant for this dissociation.

Tyrosine hydroxylase; Phosphorylation; cyclic AMP; Catecholamine; Adrenaline; Noradrenaline

1. INTRODUCTION

Tyrosine 3-monooxygenase (tyrosine hydroxylase (TH); EC 1.14.16.2) has recently received much attention due to its key function in catecholamine biosynthesis and also its possible role in the pathogenesis of various neurological and psychiatric disorders [1,2]. Thus, detailed information on the catalytic mechanism and regulatory properties of the enzyme, as well as its molecular biology, is required in order to understand the modulation of neurotransmitter biosynthesis in the central nervous system in health and disease.

The enzyme has been shown to be subject to a number of regulatory mechanisms in vivo. Particular emphasis has been put on long-term regulation at the level of gene expression and short-term regulation by phosphorylation/dephosphorylation [3,4]. The feed-back inhibition of TH in vitro by dopamine, noradrenaline (NA) and adrenaline has also been proposed to be of physiological significance [3,5,6], but the mechanism of this inhibition has so far not been unambiguously identified. Recently, we have presented evidence that catecholamines control the activity of TH in vitro by a direct coordination to iron at the active site [7,8]. However, little is yet known about the structural requirements for this association, the affinity of binding, the rates of association/dissociation and its physiological significance.

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Abbreviations: TH, tyrosine hydroxylase; NA, noradrenaline

2. EXPERIMENTAL

2.1. *Materials*

The catalytic subunit of bovine heart cyclic AMP-dependent protein kinase was purified according to [9]. [γ-^{32}P]ATP and [^{3}H]NA were from Amersham International, England. Sephadex G-25 was from Pharmacia (Uppsala, Sweden).

2.2. *TH purification and phosphorylation*

Bovine and rat adrenal TH were purified as described [7]. The phosphorylation of bovine TH was performed at 30°C in a mixture containing 0.1 mg/ml TH, 30 μg/ml catalytic subunit of bovine heart cyclic AMP-dependent protein kinase, 6.25 mM $Mg(acetate)_2$, 25 mM NaHepes, pH 7.0, 1 mM ATP, 1.25 mM dithiothreitol and 1 mM EDTA. Similar experiments performed in the presence of [γ-^{32}P]ATP demonstrated that 0.6–0.7 mol phosphate/mol enzyme subunit was incorporated into TH under these conditions.

2.3. *Assay of catecholamine release from TH*

The release of catecholamines from TH was assayed by rapid ultrafiltration. The enzyme (2.5 μM subunit) was incubated at either 4 or 30°C as described below. After incubation, 100 μl of the enzyme was added to Ultrafree-MC low binding cellulose ultrafiltration units (type 10000 NMWL) from Millipore Corp. (Bedford, MA). Free and bound catecholamines were separated by centrifugation of the samples (4 min at 3000 × g_{av}). The catecholamines in the ultrafiltrate (14 μl) were analyzed on a Hewlett-Packard 60 × 4.6 mm internal diameter (3 μM particle size) C_{18} reversed phase column, equipped with a precolumn and equilibrated with 10 mM sodium phosphate pH 3.9, 25% methanol (v/v) and 0.007% sodium dodecylsulfate. The flow-rate was 0.7 ml/min and an LKB 2134 electrochemical detector was used for detection.

Alternatively, the endogenous catecholamines were first removed by incubation at pH 4.4 at 4°C for 15 min, followed by ammonium sulfate precipitation (50%), incubation with 0.1 μM [^{3}H]NA at pH 7.5 and gel filtration (Sephadex G-25) at this pH. The release of [^{3}H]NA from the complex was assayed by ultrafiltration as described above.

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3. RESULTS AND DISCUSSION

3.1. *pH-dependent release of catecholamines from TH*

The bovine adrenal enzyme as isolated contains tightly bound NA and adrenaline, i.e. about 0.35 mol catecholamine/mol subunit [8]. At neutral pH and 4°C, the amines are released very slowly, but the rate is markedly increased below pH 6.0 (fig.1A). When the endogenous catecholamines are removed by ammonium sulfate precipitation at pH 4.4, the enzyme rebinds labelled NA with high affinity at pH 7.0. The dissociation of the TH-[^{3}H]NA complex revealed the same pH dependence as for the enzyme as isolated, with an 8-fold increase from pH 6.0 to pH 4.0 (fig.1B). The pseudo-first order rate constant for the dissocation increased by a factor of 27 from pH 6.0 (k_{-1} = 3.4 × 10^{-5} s^{-1}) to pH 4.8 (k_{-1} = 9.0 × 10^{-4} s^{-1}). The rates did not change when measured in the presence of excess (0.3 mM) unlabelled NA (data not shown). From fig.1B we can infer the nature of at least one of the bonds involved in this tight binding, i.e. a group with an apparent pK_a of about 5.3 at 4°C. This value points to a carboxyl group involved in electrostatic interaction (forming a salt bridge) with the protonated amino group of NA (and other catecholamines). Our resonance Raman spectroscopic studies have revealed additional charge transfer interactions between catecholamines and the high-spin (S = 5/2) Fe(III) [8]. This indicates that the distance for this interaction is of the same order as determined for the Fe(III)-oxyanion bond length in the [Fe(salen)catechol]$^-$ model complex (i.e. 1.8 Å) [10].

The pH-dependent release of catecholamines from TH is not restricted to the bovine adrenal enzyme. Very similar titration curves to those presented in fig.1 were obtained with partially purified rat adrenal TH (results not shown). Furthermore, rat pheochromocytoma TH contains dopamine and NE which is released by acid treatment [11]. It has also been reported that the rat striatal enzyme is inhibited by dopamine and activated by brief acid treatment [12,13]. It was suggested that dopamine was 'acting as a kind of catalyst', since they were unable to observe any direct binding of the amine to the enzyme [13]. Our results (fig.1), however, support the conclusion that this acid activation of rat striatum TH should rather be explained in terms of a reversible dissociation of enzyme-bound catecholamines.

3.2. *Effect of phosphorylation of Ser-40 on the release of catecholamines*

We have recently shown that bovine adrenal TH is selectively phosphorylated at Ser-40 by cyclic AMP-dependent protein kinase (EC 2.7.1.37) [14] and that this phosphorylation is accompanied by an increased enzymatic activity and altered spectroscopic properties of the catecholamine-Fe(III) complex at the active site

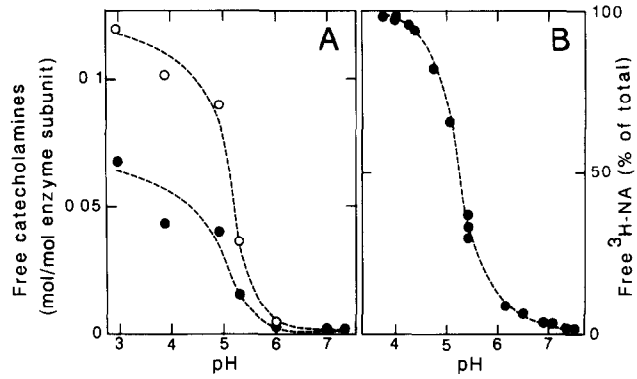

Fig.1. Effect of pH on the release of catecholamines from bovine adrenal TH. (A) Release of endogenous NE (●) and adrenaline (○) from the enzyme as isolated. (B) Release of bound [^{3}H]NE from the enzyme after removal of endogenous catecholamines and binding of [^{3}H]NE. The TH concentration was 0.15 mg/ml (2.5 μM subunit) and the amount of catecholamines released after incubation for 30 min at 4°C in 50 mM sodium acetate buffer was measured.

[15,16]. From fig.2 it is seen that the dissociation of the TH-[^{3}H]NA complex at pH 7.0 and 30°C follows pseudo-first order kinetics, with a rate constant of k_{-1} = 8.7 × 10^{-5} s^{-1}, and that phosphorylation causes a 6-fold increase in the rate constant (k_{-1} = 5.1 × 10^{-4} s^{-1}). At acidic pH values, the effect of phosphorylation is less pronounced (data not shown).

These findings explain our recent observations that phosphorylation of Ser-40 activates the enzyme by facilitating the reduction of the enzyme-bound Fe(III) by the cofactor [16]. Thus, catecholamines have been shown to be competitive inhibitors of this cofactor [17] and phosphorylation efficiently reverses this inhibition.

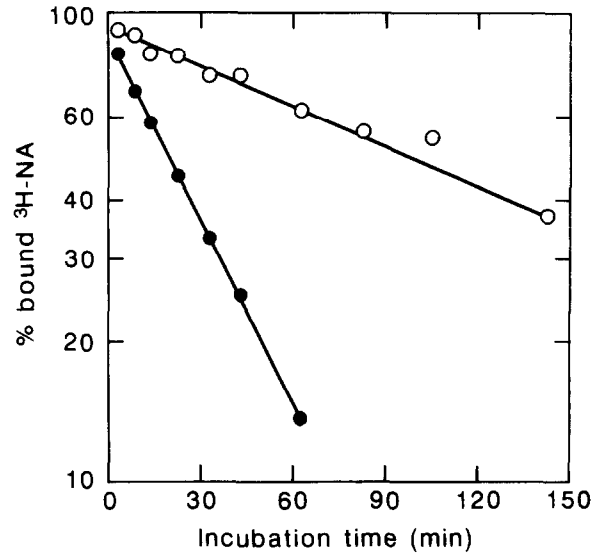

Fig.2. The effect of phosphorylation of Ser-40 on the rate of [^{3}H]NE release from TH. TH was phosphorylated as described in section 2.2 (●). The control enzyme was incubated under identical conditions, but without Mg(acetate)$_2$ (○). Aliquots were taken out at intervals and free and bound [^{3}H]NA were determined as described in section 2.3.

3.3. *Conclusions*

Our findings have also other obvious implications for the interpretation of in vitro studies on TH. First, for the crude enzyme or enzyme isolated by published procedures, the active form represents only a fraction of the total, while the rest is inactive due to tightly bound catecholamines [7,8]. Secondly, the pH of the assay may also affect the concentration of active enzyme. Thus, different pH optima have been found for the activity of the enzyme isolated with bound catecholamines, for acid-treated enzyme and for phosphorylated enzyme [17]. Finally, the stability of the catecholamine-coordinated enzyme towards inactivation seems to be higher than for the free enzyme [13].

Based on estimates of the K_d value determined by equilibrium dialysis (i.e. $0.1–0.2 \times 10^{-6}$ M at pH 7.0 and 4°C) [18], it is evident that the dissociation (*off*) rate is several orders of magnitude slower than the *on* rate. The decrease in affinity by phosphorylation of Ser-40 seems to be brought about mainly by a 6-fold increase in the *off* rate (fig.2). Our data imply that the inhibition of TH by physiological intracellular concentrations of catecholamines (i.e. in the range of 0.5–1.0 μM) [19] is likely to be a sustained effect also under in vivo conditions. The dissociation rates found for the TH-[^{3}H]NA complex in vitro are in the same range as reported for the dissociation of the complex of cyclic AMP and the regulatory subunits of protein kinase A [20], and changes in these rate constants may have profound physiological effects. Taken together, our findings point to a key role of catecholamines in the regulation of their biosynthesis and activation of TH by phosphorylation at least partly is to be explained by a decreased affinity for inhibitory catecholamines.

The inhibition of TH in vivo may be promoted by high intracellular levels of catecholamines, either as a result of physiological responses or induced pharmacologically. Thus, it may partly explain the side effects and fluctuations in the clinical response observed in long-term treatment of Parkinson's disease with the dopamine precursor L-DOPA (3,4-dihydroxyphenylalanine) [21]. In fact, the inhibition of TH would not be limited to the dopaminergic system, but would also include the noradrenergic neurons, and tryptophan hydroxylase may be inhibited by a similar mechanism [22]. A slow *off* rate for inhibitors of TH other than catecholamines, e.g. structurally related drugs [23], may also contribute to their sustained pharmacological effects.

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REFERENCES

[1] Kidd, K.K., Kidd, J.R., Pakstis, A.J., Castiglione, C.M., Egeland, J.A. and Mallet, J. (1989) in: Molecular Probes: Technology and Medical Applications (Albertini, A. et al. eds) Raven, New York.
[2] Mallet, J., Boni, C., Dumas, S., Darmon, M., Biguet, N.F., Grima, B., Horellou, P. and Lamoroux, A. (1987) J. Psychiatr. Res. 21, 559–568.
[3] Kaufman, S. and Kaufman, E.S. (1985) in: Folates and Pterins, vol.2 (Blakeley, R.L. and Benkovic, S.J. eds) pp.251–352, Wiley, New York.
[4] Zigmond, R.E., Schwarzschild, M.A. and Rittenhouse, A.R. (1989) Annu. Rev. Neurosci. 12, 415–461.
[5] Udenfriend, S., Zatzman-Niremberg, P. and Nagatsu, T. (1965) Biochem. Pharmacol. 14, 837–845.
[6] Mann, S.P. and Gordon, J.I. (1979) J. Neurochem. 33, 133–138.
[7] Haavik, J., Andersson, K.K., Petersson, L. and Flatmark, T. (1988) Biochim. Biophys. Acta 953, 142–156.
[8] Andersson, K.K., Cox, D.D., Que, L. jr, Flatmark, T. and Haavik, J. (1988) J. Biol. Chem. 263, 18621–18626.
[9] Reimann, E.M. and Beham, R.A. (1983) Methods Enzymol. 99, 51–55.
[10] Heistand, R.H. ii, Roe, A.L. and Que, L. jr (1982) Inorg. Chem. 21, 676–681.
[11] Andersson, K.K., Haavik, J., Que, L. jr, Flatmark, T., Thibault, J. and Petersson, L. (1989) J. Inorg. Biochem. 36, 323.
[12] Okuno, S. and Fujisawa, H. (1985) J. Biol. Chem. 260, 2633–2635.
[13] Fujisawa, H. and Okuno, S. (1986) in: Amino Acids in Health and Disease: New Perspectives (Kaufman, S. ed.) pp.245–266, Alan R. Liss, New York.
[14] Haavik, J., Schelling, D.L., Campbell, D.G., Andersson, K.K., Flatmark, T. and Cohen, P. (1989) FEBS Lett. 251, 36–42.
[15] Haavik, J., Andersson, K.K., Flatmark, T. and Petersson, L. (1989) Pteridines 1, 11–16.
[16] Andersson, K.K., Haavik, J., Martínez, A., Petersson, L. and Flatmark, T. (1989) FEBS Lett. 258, 9–12.
[17] Lazar, M.A., Lockfeld, A.J., Truscott, R.J.W. and Barchas, J.D. (1982) J. Neurochem. 36, 409–422.
[18] Martínez, A., Andersson, K.K., Haavik, J. and Flatmark, T. (1989) Abstr. Commun. FEBS Meet. Tu, 132.
[19] Perlman, R.L. and Sheard, B. (1982) Biochim. Biophys. Acta 719, 334–340.
[20] Øgreid, D. and Døskeland, S.O. (1981) FEBS Lett. 129, 282–286.
[21] Quinn, N.P. (1987) in: Handbook of Parkinson's Disease (Koller, W.C. ed.) pp.317–337, Marcel Dekker, New York.
[22] Koe, B.K. (1971) Fed. Proc. 30, 886–896.
[23] Lloyd, T., Boyd, B., Walega, M.A., Jones Ebersole, B. and Weisz, J. (1982) J. Neurochem. 38, 948–954.