Volume 258, number 1, 9-12 FEB 07819 November 1989

Evidence from EPR spectroscopy that phosphorylation of Ser-40 in bovine adrenal tyrosine hydroxylase facilitates the reduction of high-spin Fe(III) under turnover conditions

Kristoffer K. Andersson, Jan Haavik, Aurora Martinez, Torgeir Flatmark and Leif Petersson*

Department of Biochemistry, University of Bergen, Bergen, Norway and **Department of Biophysics, University of Stockholm, Stockholm, Sweden*

Received 15 September 1989: revised version received 27 September 1989

The tetrahydrobiopterin-dependent iron-enzyme tyrosine hydroxylase (TH) catalyses the rate-limiting step in catecholamine biosynthesis. Electron paramagnetic resonance (EPR) data show that following phosphorylation of Ser-40 by protein kinase A, the enzyme-bound Fe(III), coordinated to catecholamines, can be reduced by 6-methyl-tetrahydropterin under turnover conditions. The 8-fold increase in product formation upon phosphorylation can partly be explained by an increase in the fraction of active TH, by dissociation of the endogenous catecholamine inhibitors.

Tyrosine hydroxylase; Phosphorylation; Electron paramagnetic resonance; Cyclic AMP-dependent protein kinase; Non-heme iron; Catecholamine; Pteridine; (Bovine adrenal)

1. INTRODUCTION

Tyrosine 3-monooxygenase (tyrosine hydroxylase, TH, EC 1.14.16.2) catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the rate-limiting step in the biosynthesis of catecholamines (neurotransmitters or hormones) [1]. The mammalian tyrosine, phenylalanine and tryptophan hydroxylases constitute a group of enzymes which all require iron and a tetrahydropterin cofactor for catalytic activity [2,3]. In their C-terminal and presumed catalytic domains, they have large amino acid sequence homology [4,5].

All three hydroxylases are activated in vivo and in vitro by phosphorylation [3,6,7]. The combination of phosphorylation/dephosphorylation and feedback inhibition (by catecholamines in the case of TH [8]) may represent a major short-term (in minute time scale) regulatory mechanism [9]. In bovine adrenal TH [10] and rat pheochromocytoma TH [11,12], cAMP-dependent protein kinase phosphorylates Ser-40 specifically, without phosphorylation of other potential sites (e.g. Ser-8 and Ser-19). Furthermore, in isolated bovine adrenal chromaffin cells, cAMP-dependent phosphorylation increases the conversion of tyrosine to DOPA by a factor of 3 in situ [13], and inhibition of protein phosphatase 2A increases TH activity by about 80% [10].

Highly purified bovine adrenal TH contains significant amounts of iron (0.7 atoms per subunit) and has a high spin (S = 5/2) Fe(III) EPR signal [14,15]. This EPR signal correlates with an unusual blue-green colour, which is due to a Fe(III)-catecholamine complex [16,17]. The present study was performed to gain more information on the catalytic reaction mechanism, the functional role of the associated feedback inhibitors adrenaline and noradrenaline (0.24 and 0.11 per enzyme subunit, respectively), and their relation to the activation of TH by phosphorylation of Ser-40.

Correspondence (present) address: K.K. Andersson, Deparment of Biochemistry, Medical School, University of Minnesota, Minneapolis, MN 55455, USA

Abbreviations: TH, tyrosine hydroxylase; EPR, electron paramagnetic resonance; DOPA, 3,4-dihydroxyphenylalanine; HPLC, high-performance liquid chromatography; 6-MPH_4, 6-methyltetrahydropterin

2. EXPERIMENTAL

2.1. *Tyrosine hydroxylase and EPR measurements*

Bovine adrenal TH was purified as described [15]; the specific activity was typically 350-400 nmol DOPA/min per mg protein at pH 6.0 in the presence of 1 mM dithiothreitol, 0.1 mg catalase/ml and 0.2 M Mes [15]. The formation of DOPA was determined by HPLC [18]. The TH activity was also assayed at pH 7.2 (incubation in the EPR tubes) in 0.2 M KCl, 0.1 M K-phosphate and 0.1 mg catalase/ml. Measurement of EPR spectra and sample handling were performed as before [15,19] using a Bruker ESP 300 EPR system with an Oxford Instruments helium flow cryostat (ESR-9).

2.2. *Phosphorylation of TH*

The catalytic subunit of bovine heart cAMP-dependent protein kinase was purified according to [20]. The cAMP-dependent phosphorylation of TH was performed at 30°C for 15 min, in a reaction mixture containing 0.2 M KCl, 75 mM K^+-phosphate (pH 7.0),

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00145793/89/$3.50

1 μM dithiothreitol, 5 mM Mg^{2+}(acetate)$_2$, 2 mg TH/ml, 1 mM [^{32}P]-ATP and 0.1 mg/ml of catalytic subunit of bovine heart cAMP-dependent protein kinase, and the phosphorylation was stopped by incubation at 4°C. After phosphorylation, ATP was rapidly removed by gel filtration [19]. In identical parallel control experiments, the reaction mixture did not contain ATP. The amount of phosphate incorporated was determined by spotting aliquots on phosphocellulose strips [7,21] or by isolating the phosphorylated proteins by sodium dodecyl sulfate electrophoresis, and the incorporated ^{32}P was determined by liquid scintillation counting. It should be mentioned that some preparations of the enzyme contained some protein phosphatase 2A, which dephosphorylated the enzyme [10], and in this case, the enzyme behaved as in the control samples.

3. RESULTS

3.1. *Effects of phosphorylation at Ser-40 on the EPR spectrum of bovine adrenal tyrosine hydroxylase under turnover conditions*

Our previous EPR studies on bovine adrenal TH [15], with one of the highest reported specific enzymatic activities, have shown the presence of high-spin Fe(III), in a slightly distorted axial symmetry giving an EPR signal with g values around 7.0, 5.2 and 1.9 ($|E/D| = 0.04$). This EPR signal dit not change significantly in the presence of substrates or cofactor, added alone or in combination [15]. In contrast, after phosphorylation of the enzyme at Ser-40, by the catalytic subunit of c-AMP-dependent protein kinase [10], large changes in the EPR spectrum were observed under turnover conditions (fig.1). The covalent binding of 0.6–0.8 mol phosphate per mol of enzyme subunit, results in a nearly identical EPR spectrum (fig.1B) as for the enzyme as isolated (fig.1A). Computer subtraction, however, revealed an approximately 30% decrease in the intensity of the axial type signal at 3.6 and 7 K. No major effect on the microwave saturation behavior of the EPR signal was observed at 3.6 K and 7 K upon phosphorylation (data not shown).

On addition of 1.5 mM L-tyrosine and incubation for 1 min at 25°C in the EPR tube, the EPR spectrum at 3.6 K revealed an 80 ± 8% (5 different samples) decrease of the axial type EPR signal for an enzyme containing 0.6–0.8 incorporated phosphate per subunit (fig.1C). This effect was less pronounced for enzyme preparations with < 0.5 incorporated phosphate per subunit (data not shown). Concomitant with this decrease, a new EPR signal, with g values at 9.2, 4.8 and 3.7, appears (fig.1C) [19,22]. We suggest that this signal originates form high-spin Fe(III) in an environment of distorted rhombical symmetry ($|E/D| = 0.22$–0.25). The observed g values of 4.8 and 3.7 orginate from the middle Kramers doublet and the associated spectral features are more clearly seen at higher microwave power (data not shown). Furthermore, also the isotropic $g = 4.3$ signal increases after addition of tyrosine (fig.1C). Control experiments with the non-phosphorylated enzyme incubated with 1.5 mM L-tyrosine (9 different samples), showed only a minimal (4 ± 2.5%) decrease of the axial type EPR signal. Similar results were obtained with preparations containing protein phosphatase activity (data not shown).

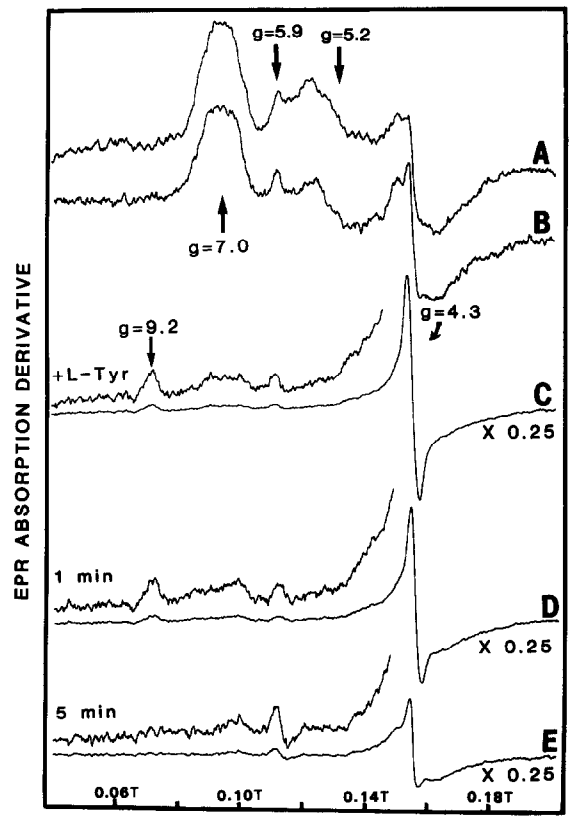

Fig.1. Effects of phosphorylation of Ser-40, substrate and cofactor on EPR spectra of bovine adrenal TH. EPR spectra at 3.6 K, 0.24 mW microwave power, 10 G modulation, 9.23 GHz, 4 accumulations of: (A) Enzyme as isolated (3 mg/ml) at pH 7.2; (B) After phosphorylation of Ser-40 (0.6–0.8 incorporated phosphate per enzyme subunit) by catalytic subunit of cAMP-dependent protein kinase; (C) Sample B after additional incubation with 1.5 mM L-tyrosine for 1 min at 25°C; (D) Sample C after additional incubation with 2 mM 6-MPH$_4$ for 1 min at 25°C; and (E) Sample D after extra 4 min incubation at 25°C. All spectra have been corrected for dilutions.

Aerobic incubation of the phosphorylated enzyme (50 μM of subunit TH) at 25°C in the EPR tubes with 1.5 mM L-tyrosine and 2 mM 6-MPH$_4$ (no dithiothreitol) resulted in time-dependent changes in the EPR spectrum (fig.1D,E). The EPR signals with $g_{max} = 7.0$ ($|E/D| = 0.04$) and $g_{max} = 9.2$ ($|E/D| = 0.22$–0.25) virtually disappeared. During the incubation period, the enzyme was catalytically active, i.e. turnover conditions were present. After 5-min incubation (pH controlled to be between 7.3 and 7.1), about 59 nmol (390 μM) DOPA had been formed in the EPR tubes. DOPA formation, by the same incubation mixture at 25°C, continued for at least 7 additional minutes, until the cofactor had been consumed. Control experiments under identical turnover conditions

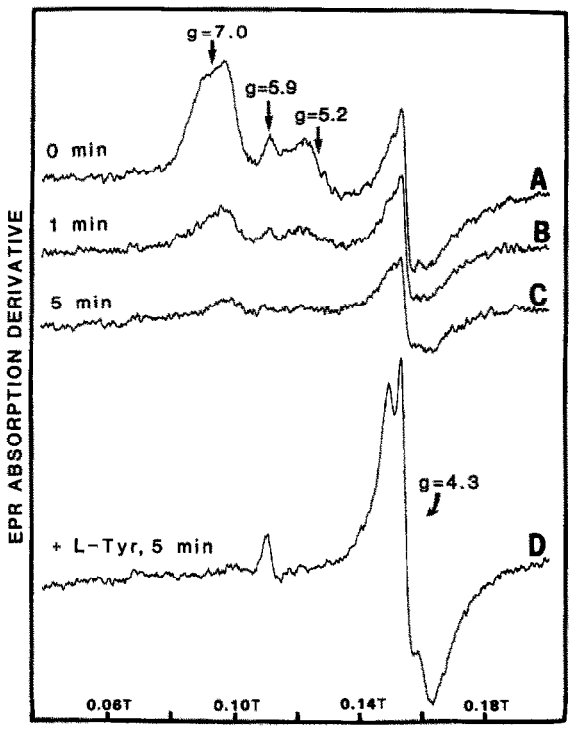

Fig.2. EPR spectra of phosphorylated bovine TH; reduction of the enzyme with cofactor without substrate and effect of added substrate to the reduced enzyme. EPR conditions as in fig.1. (A) Resting phosphorylated (0.6–0.8 phosphate incorporated per subunit) TH (3 mg/ml) at pH 7.2; (B) Sample A with 2 mM 6-MPH_4 and incubation for 1 min at 25°C; (C) Sample B after additional 4-min incubation at 25°C; and (D) Sample C with 1.5 mM L-tyrosine and 5 min incubation at 25°C.

with the enzyme as isolated or containing protein phosphatase, showed a less than 25% decrease in the axial type EPR signal (data not shown), while the DOPA formed was a factor 8 lower.

3.2. *Phosphorylation of TH facilitates the reduction of the enzyme-bound (Fe)III by the cofactor*

The effect of aerobic incubation of the resting phosphorylated TH (fig.2A) with 2 mM 6-MPH_4 at 25°C, in the absence of natural substrate or other reductants, is shown in fig.2B,2C. After 1-min incubation with 6-MPH_4, a significant decrease (55 ± 15%) was seen in the axial type EPR signal (fig.2B) and no new signal appeared. After 5-min incubation, the phosphorylated enzyme was almost EPR silent (fig.2C). These large spectral changes could not be seen for the enzyme as isolated and for enzyme preparations containing protein phosphatase activity. In these cases, only a maximum of 10% decrease of the axial type EPR signal could be observed (data not shown). On further incubation with 1.5 mM L-tyrosine for 5 min at 25°C, a new rhombic type EPR signal from high-spin Fe(III) appeared, with g values centered around 4.3 (fig.2D). This signal, although less pronounced, is also seen in fig.1B and E. Nearly 66 nmol (440 μM) DOPA was formed in this experiment within the 5-min incubation period, and the enzyme was still active.

4. DISCUSSION

4.1. *The oxidation state of the enzyme-bound iron during catalytic turnover*

The reaction mechanisms for the pteridine-dependent hydroxylases are not clear. In particular, the role of iron and its redox state are not settled, although a ferrous EPR-silent form has been suggested to be involved in catalysis [15,17,19,23–25]. The EPR active Fe(III) in bovine adrenal TH, with g_{max} = 7.0, is correlated to the Fe(III) that is coordinated to adrenaline/noradrenaline [15,17]. Quantitation of the tightly bound catecholamines in the enzyme as isolated indicates that about half of the total enzyme-bound iron is coordinated to the feedback inhibitors [17], and the other half of the iron could be responsible for the enzymatic activity. In rat pheochromocytoma TH dopamine is the major Fe(III) coordinated catecholamine [26]. The bidentate binding of catecholate to Fe(III) in TH has been considered to lower the redox potential of this iron [17]. Its EPR properties did not significantly change during turnover conditions, or in the presence of the cofactor, indicating that the Fe(III) species was not part of the catalytic cycle [15]. By contrast, for phosphorylated TH, the EPR signals with g_{max} = 7.0 (enzyme as isolated) and g_{max} = 9.2 (induced by L-tyrosine) almost completely disappeared during turnover conditions (figs.1E and 2D). Also, the presence of cofactor alone, resulted in an almost EPR silent enzyme (fig.2C). Our interpretation of these results is that phosphorylation of TH facilitates the reduction of Fe(III) to Fe(II) by the cofactor. The reduction is associated with an 8-fold increase of the DOPA formation. Our results are compatible with the Fe(II) form being part of the catalytic cycle, in agreement with models for phenylalanine hydroxylase [24] and rat TH [25]. For the phosphorylated bovine TH, we have also shown that the addition of the L-enantiomer of tyrosine ([19,22] and fig.1C) induces a new Fe(III) EPR signal (g_{max} = 9.2). A similar substrate effect has been observed for phenylalanine hydroxylase [24]. Thus, the Fe(III) present in phosphorylated TH behaves partially as the Fe(III) present in phenylalanine hydroxylase, in agreement with light absorption and resonance Raman spectroscopic data [17,26].

The appearance of the new rhombic type EPR signal, centered around g = 4.3 (fig.2D), may have alternative explanations. The possibility that this iron is catalytically active is now being considered. From knowledge about the general EPR characteristics of Fe(III), it is possible to estimate that the new isotropic EPR signal in fig.2D or the increase in the g = 4.3 signal (fig.1C), should not represent a large proportion of Fe(III) compared to the amounts of Fe(III) (g_{max} = 7.0 and 9.2)

that can be reduced with 6-MPH$_4$. Furthermore, if one assumes only small differences in D-values, as reported for phenylalanine hydroxylase [27], then the two Fe(III) signals with g_{max} = 7.0 and 9.2 should represent similar and substantial amounts of Fe(III); see simulation of phenylalanine hydroxylase spectra [28].

4.2. *The molecular mechanism of the activation by phosphorylation*

The effect of phosphorylation of TH, permitting previously inhibited iron to become reduced and enzymatically active, could have several explanations. Most likely, phosphorylation increases the dissociation of catecholamines, as indicated by the 30% decrease in the axial type EPR signal (fig.1B). As catecholamines are competitive inhibitors to the cofactor [29,30], a decrease in their affinity to TH would permit reduction of the Fe(III) by the cofactor.

The activity of TH has been reported to increase up to 16-fold on phosphorylation, when assayed at pH 7.2 [3]. This activation has generally been considered to be due to the lowering of the K_m value for the cofactor (i.e. from 0.5 mM to 0.2 mM for 6-MPH$_4$ [29])[1] and a basic shift of the pH-activity curve (pH optimum increases from 6.0 to 7.4). Furthermore, when catecholamines are dissociated by acid precipitation of rat brain TH, phosphorylation by itself results in a modest activation [31,32]. Recently, in an esthetically nice model, it has been proposed that phosphorylation permits the cofactor to make new hydrogen bonds with the enzyme [33]. As demonstrated in this work, models explaining the large activation of TH by phosphorylation should also include the contribution of dissociation of tightly bound catecholamines from the enzyme.

Acknowledgements: We thank Ms S.E. Riise and Mr T. Astlind for expert technical assistance and Dr J. Thibault at College de France, Paris for valuable discussions. This work was supported by grants from Nordisk Industrifond, Nordiska forskarkurser, the Norwegian Research Council for Science and the Humanities and the Swedish Natural Science Research Council.

REFERENCES

[1] Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) J. Biol. Chem. 239, 2910–2917.
[2] Kaufman, S. and Fisher, D. (1974) in: Molecular Mechanisms of Oxygen Activation (Hayaish, O. ed.) pp. 285–369, Academic Press, New York.
[3] Kaufman, S. and Kaufman, E.S. (1985) in: Folates and Pterins, vol. 2 (Blakley, R.L. and Benkovic, S.J. eds) pp. 251–352, John Wiley and Sons, New York.
[4] Ledley, F.D., DiLella, A.G., Kwok, S.C.M. and Woo, S.L.C. (1985) Biochemistry 24, 3389–3394.
[5] Grenett, H.E., Ledley, F.D., Reed, L.L. and Woo, S.L.C. (1987) Proc. Natl. Acad. Sci. USA 84, 5530–5534.
[6] Morgenroth, V.H., Hegstrand, L.R., Roth, R.H. and Greengard, P. (1975) J. Biol. Chem. 250, 1946–1948.
[7] Døskeland, A.P., Døskeland, S.O., Øgreid, D., and Flatmark, T. (1984) J. Biol. Chem. 259, 11242–11248.
[8] Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965) J. Pharmacol. Exp. Ther. 148, 1–8.
[9] Joh, T.H., Park, D.H. and Reis, D.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4744–4748.
[10] Haavik, J., Schelling, D.L., Campbell, D.G., Andersson, K.K., Flatmark, T. and Cohen, P. (1989) FEBS Lett. 251, 36–42.
[11] Campbell, D.G., Hardie, D.G., and Vulliet, P.R. (1986) J. Biol. Chem. 261, 10489–10492.
[12] Pigeon, D., Ferrara, P., Gros, F. and Thibault, J. (1987) J. Biol. Chem. 262, 6155–6158.
[13] Waymire, J.C., Johnston, J.P., Hummer-Lickteig, K., Lloyd, A., Vigny, A. and Craviso, G.L. (1988) J. Biol. Chem. 263, 12439–12447.
[14] Haavik, J., Andersson, K.K., Petersson, L. and Flatmark, T. (1986) in: Chemistry and Biology of Pteridines 1986. Pteridines and Folic Acid Derivatives, Proceedings of the 8th International Symposium (Cooper, B.A. and Whitehead, V.M. eds), pp. 201–204, de Gruyter, Berlin.
[15] Haavik, J., Andersson, K.K, Petersson, L. and Flatmark, T. (1988) Biochem. Biophys. Acta 953, 142–156.
[16] Andersson, K.K., Cox, D.D., Que, L. jr., Petersson, L., Flatmark, T. and Haavik, J. (1987) Rec. J. R. Nethl. Chem. Soc. 106, 244.
[17] Andersson, K.K., Cox, D.D., Que, L. jr., Flatmark, T. and Haavik, J. (1988) J. Biol. Chem. 263, 18621–18626.
[18] Haavik, J. and Flatmark, T. (1980) J. Chromatogr. 198, 511–515.
[19] Haavik, J., Andersson, K.K, Petersson, L. and Flatmark, T. (1989) Pteridines 1, 11–16.
[20] Reimann, E.M. and Beham, R.A. (1983) Methods Enzymol. 99, 51–55.
[21] Roskoski, R. jr. (1983) Methods Enzymol. 99, 3–6.
[22] Andersson, K.K, Haavik, J., Flatmark, T. and Petersson, L. (1988) Abstr. Commun. Meet. Internat. Union of Biochem., Prague, vol. 4, p. 102.
[23] Wallick, D.E., Bloom, L.M., Gaffney, B.J. and Benkovic, S.J. (1984) Biochemistry 23, 1295–1302.
[24] Benkovic, S., Wallick, D., Bloom, L., Gaffney, B.J., Domanico, P., Dix, T. and Pember, S. (1985) Biochem. Soc. Trans. 13, 436–438.
[25] Dix, T.A., Kuhn, D.M. and Benkovic, S.J. (1987) Biochemistry 26, 3354–3361.
[26] Andersson, K.K., Haavik, J., Que, L. jr., Flatmark, T., Thibault, J. and Petersson, L. (1989) J. Inorg. Biochem. 36, 323.
[27] Bloom, L.M., Benkovic, S.J. and Gaffney, B.J. (1986) Biochemistry 25, 4204–4210.
[28] Yang, A.-S. and Gaffney, B.J. (1987) Biophys. J. 51, 55–67.
[29] Lazar, M.A., Lockfeld, A.J., Truscott, R.J.W. and Barchas, J.D. (1982) J. Neurochem. 39, 409–422.
[30] Fitzpatrick, P.F. (1988) J. Biol. Chem. 263, 16058–16062.
[31] Okuno, S. and Fujisawa, H. (1985) J. Biol. Chem. 260, 2633–2635.
[32] Fujisawa, H. and Okuno, S. (1987) in: Amino Acids in Health and Disease: New Perspectives (Kaufman, S. ed.) pp. 245–266, Alan R. Liss, New York.
[33] Bailey, S.W., Dillard, S.B., Thomas, K.B. and Ayling, J.E. (1989) Biochemistry 28, 494–504.

[1] In our experimental conditions, a high concentration of the cofactor (2 mM of 6-MPH$_4$) was used; i.e. at a concentration four times higher than the reported K_m value. So, our observations were not only reflecting the low affinity for the cofactor for the enzyme as isolated.