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Evidence for a functionally important histidine residue in human tyrosine hydroxylase

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Summary. Recombinant human tyrosine hydroxylase isozyme 1 (hTH1) shows a time- and concentration-dependent loss of catalytic activity when incubated with diethylpyrocarbonate (DEP) after reconstitution with Fe(II). The inactivation follows pseudo-first order kinetics with a second order rate constant of $300\,M^{-1}\cdot min^{-1}$ at pH 6.8 and 20°C and is partially reversed by hydroxylamine. The difference absorption spectrum of the DEP-modified vs native enzyme shows a peak at 244 nm, characteristic of mono-N-carbethoxyhistidine. Up to five histidine residues are modified per enzyme subunit by a five-fold excess of the reagent, and two of them are protected from inactivation by the active site inhibitor dopamine. However, derivatization of only one residue appears to be responsible for the inactivation. Thus, no inactivation by DEP was found when the apoenzyme was preincubated with this reagent prior to its reconstitution with Fe(II), modifying four histidine residues.

Keywords: Amino acids – Human tyrosine hydroxylase – Histidine – Chemical modification – Diethylpyrocarbonate

Abbreviations: BH_4, (6R)-l-*erythro*-tetrahydrobiopterin; DEP, diethylpyrocarbonate; DOPA, 3,4-dihydroxyphenylalanine; hTH1, human tyrosine hydroxylase isoenzyme 1; apo-hTH1, apoenzyme of hTH1; Fe(II)-hTH1, holoenzyme (iron reconstituted) of hTH1; dopamine-Fe(III)-hTH1, holoenzyme of hTH1 with dopamine bound; TH, tyrosine hydroxylase.

Introduction

Tyrosine hydroxylase (TH, EC 1.14.16.2) catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) which is the first step in the biosynthesis of catecholamine neurotransmitters (Levitt et al., 1965). TH is a non-heme iron and tetrahydrobiopterin dependent enzyme and the four human isoforms (hTH1–hTH4) are all overexpressed in *E. coli* (Haavik et al.,

1991; Sutherland et al., 1993) as tetrameric apoenzymes (apo-hTH1 to hTH4) which are rapidly activated (up to 40 fold) by the incorporation of one equivalent of Fe(II) per subunit (Haavik et al., 1991). Together with phenylalanine and tryptophan hydroxylases, TH constitutes the superfamily of aromatic amino acid hydroxylases. Their 3-D structure is not known and although several mechanisms of hydroxylation have been proposed for these enzymes (Dix and Benkovic, 1988), the actual catalytic mechanism and residues involved in catalysis are not known. All three enzymes contain a homologous catalytic C-terminal domain, including five highly conserved histidine residues (i.e. His 191, 246, 316, 330, 335 in hTH1) (Grima et al., 1987). In this paper we present evidence by chemical modification with diethylpyrocarbonate (DEP) that the active site of human TH has at least one histidine residue, not accessible in the apoenzyme (iron free enzyme), that is essential for catalytic activity.

Materials and methods

hTH1 expression and purification

Human tyrosine hydroxylase isoform 1 (hTH1) was expressed in *E. coli* and purified to homogeneity as previously described (Haavik et al., 1991). The purified enzyme contained 0.02 ± 0.01 (mean ± S.D., $n = 4$) atoms of iron/subunit, as determined by atomic absorption spectrometry, and was considered to represent an apoenzyme (apo-hTH1). The concentration of hTH1 was determined by the absorbance at 280 nm ($\varepsilon^{1\%} = 10.4\,cm^{-1}$) at neutral pH (Haavik et al., 1988). The holoenzyme Fe(II)-hTH1 was prepared by incubation of apo-hTH1 with 0.1 mM ferrous ammonium sulphate for 5 min at 20 °C (Haavik et al., 1991, 1992) and the dopamine-Fe(III)-hTH1 complex by the incubation of Fe(II)-hTH1 with equimolar amounts of dopamine per enzyme subunit (Haavik et al., 1992).

Assay of enzyme activity

TH activity was measured as described (Reinhard et al., 1986), with 25 μM L-[3,5-^{3}H]tyrosine, 0.5 mM (6R)-l-*erythro*-tetrahydrobiopterin (BH_4), 5 mM dithiothreitol, 0.5 mg/ml catalase and 0.1 mM ferrous ammonium sulphate in 0.1 M Na-Hepes buffer pH 7.0 at 30°C. The specific activity of the purified enzyme was about 406 nmol DOPA/min/mg.

UV difference spectra

Spectroscopic studies were performed in a Hewlett-Packard 8452A Diode Array Spectrophotometer at 20°C. The reaction was initiated by the addition of 0.05–0.1 mM of an ethanolic solution of DEP to the enzyme (see figure legends). The exact concentration of the stock solution of DEP was calculated from the increase in absorbance at 230 nm (ΔA_{230}) when aliquots of this solution were added to 10 mM imidazole in 0.1 M K-phosphate, pH 7.5, using an extinction coefficient of $3000\,M^{-1} \cdot cm^{-1}$ (Melchior and Fahrney, 1970; Miles, 1977). The stoichiometry of the formation of N-carbethoxyhistidine residues was determined by the ΔA_{244}, using an extinction coefficient of $3200\,M^{-1} \cdot cm^{-1}$ (Miles, 1970).

Chemical modification and inactivation of hTH1 by DEP

The chemical modification was initiated by adding 0.05–0.5 mM of an ethanolic solution of DEP to enzyme samples (0.2–2 mg/ml) in 0.1 M K-phosphate, pH 6.8. At the indicated

time points after the addition of DEP, aliquots were withdrawn, quenched by adding 1.6 mM histidine and diluted 50-fold in distilled water prior to activity measurements. The ethanol concentration did not exceed 5% by volume and was found to have no effect on the activity or the stability of the enzyme during the incubation period. The k_{obs}-values (*pseudo*-first-order rate constants) for the inactivation at each concentration of DEP were determined from linear regression analysis of the logarithmic functions. Hydroxylamine solution was prepared by dissolving the solid reagent in 100 mM K-phosphate followed by titration to pH 6.8 with KOH.

Determination of free sulfhydryl groups

Spectrophotometric titration of thiol groups in untreated and DEP-modified protein using Ellman's reagent was carried out as described (Habeeb, 1972).

Results and discussion

UV difference spectra

When apo-hTH1 is preincubated with 0.1 mM Fe(II) at pH 6.8, the active holoenzyme containing one atom of Fe(II) per enzyme subunit is formed (Fe(II)-hTH1) (Haavik et al., 1991, 1992). The catecholamine inhibitors (dopamine, adrenaline, noradrenaline) bind with high affinity to the active site of the holoenzyme with a stoichiometry of about 1.0 mol/mol enzyme subunit (Almås et al., 1992). Dopamine binding causes the oxidation of the iron to the ferric state and a very stable complex (dopamine-Fe(III)-hTH1) is formed (Haavik et al., 1992). When the three forms of the enzyme, i.e. apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1, were incubated with DEP at pH 6.5–6.8, an increase in their absorbance between 230 and 260 nm with a maximum at 244 nm was observed (Figs. 1 and 2 and data not shown), as expected for the mono-N-carbethoxylation of histidine residues (Miles, 1977). The ΔA_{244} reached a maximum after various times of incubation, depending on the concentration of DEP. After approx. 20 min of incubation using

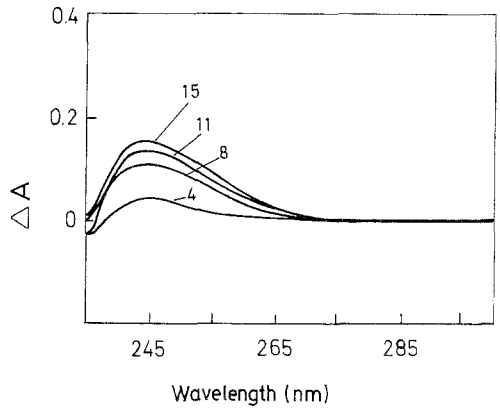

Fig. 1. UV difference spectra at three different times of incubation of apo-hTH1 with DEP. Spectra were recorded at 20°C in 0.1 M K-phosphate, pH 6.8, with hTH1 at 30 μM subunit concentration and adding 0.1 mM DEP at zero time. Incubation times are shown. The absorbance was corrected for enzyme absorption in the whole spectral range for each time of incubation

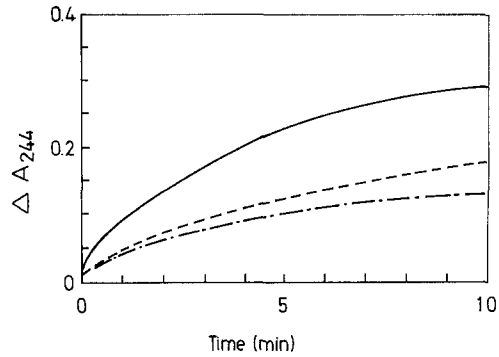

Fig. 2. Comparison of the extent of DEP incorporation in apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1. The increase in absorbance at 244 nm (ΔA_{244}) was recorded at 20°C in 0.1 M K-phosphate, pH 6.8, after adding 0.1 mM DEP at zero time to apo-hTH1 (---), Fe(II)-hTH1 (—) and dopamine-Fe(III)-hTH1 (-•-) at 20 μM subunit. For additional details, see Materials and methods

Table 1. Extent of histidine modification by DEP in apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1

Sample	Time (min)	Histidine residues modified/enzyme subunit
apo-hTH1	10	2.9
	20	4.0
Fe(II)-hTH1	10	4.0
	20	4.8
Dopamine-Fe(III)-hTH1	10	2.2
	20	2.8

The enzyme (20 μM subunit hTH1) was incubated with 0.1 mM DEP in 0.1 M K-phosphate, pH 6.8 at 20°C. For details, see Materials and methods and legend to Fig. 2. The averaged values of two experiments are shown.

0.1 mM DEP and 20 μM hTH1 subunit (5-molar excess of the reagent), the ΔA_{244} reached a maximum which corresponded to the modification of 4, 4.8 and 2.8 histidine residues/subunit for apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1, respectively (Table 1). Control experiments with ferrous ammonium sulphate and dopamine added separately or together to solutions of imidazole and DEP did not have any effect in the amount of N-carbethoxyimidazole formed. Thus, two histidine residues seem to be located at the active site since they are not accessible to DEP in the dopamine-bound holoenzyme.

Inactivation of Fe(II)-hTH1 by DEP

From the spectroscopic studies it was inferred that four histidine residues are modified in the apo-hTH1 by a five-fold excess of DEP, but DEP-modified

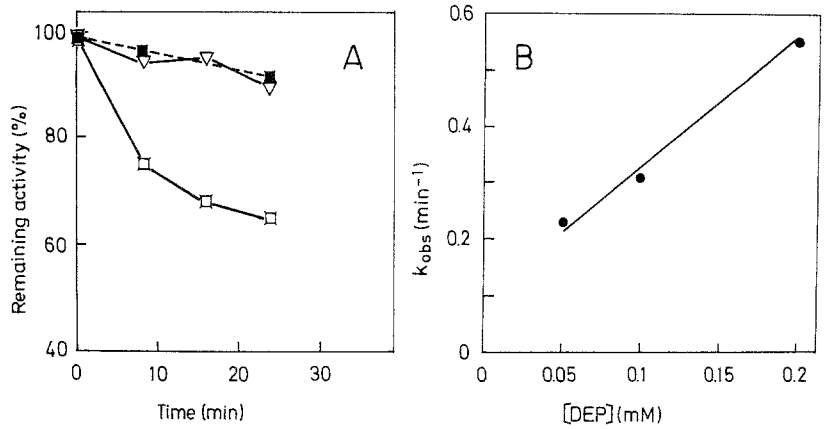

Fig. 3. Effect of DEP on the activity of hTH1. **A** The iron reconstituted holoenzyme, Fe(II)-hTH1 (10 μM subunit) was incubated at 20°C in the presence of 0.05 mM DEP (□) and in the absence of DEP (■) in 0.1 M K-phosphate, pH 6.8. Aliquots were removed at the indicated times and assayed for activity as indicated in Materials and methods. The apoenzyme, apo-hTH1, was also incubated at the same conditions as the holoenzyme in the presence of 0.05 mM DEP (▽). **B** Plot of the apparent first-order rate constants (k_{obs}) for the inactivation of Fe(II)-hTH1 at 0.05, 0.1 and 0.2 mM DEP

apo-hTH1 shows no decrease in activity as compared to the non-modified enzyme (apo- or Fe(II)-hTH1), as determined by a standard enzyme assay including 0.1 mM ferrous ammonium sulphate (Fig. 3A). Since binding of the iron to the apoenzyme is a prerequisite for activity (Haavik et al., 1991), putative iron-coordinating residues do not seem to be altered and none of the four DEP-modified histidines in apo-hTH1 appear to be essential for activity. However, if DEP is added after preincubation of the apoenzyme with Fe(II), in order to form the holoenzyme (Fe(II)-hTH1), there is a time and concentration dependent inactivation of the enzyme. The inactivation followed pseudo-first order kinetics and the k_{obs}-values were found to be proportional to the concentration of DEP with a second order rate constant of 300 $M^{-1} \cdot min^{-1}$ (Fig. 3B), indicating that the inactivation is due to a simple bimolecular reaction between the reagent and the enzyme. Since DEP reacts with L-tyrosine (see below), an appropriate study of the protection of inactivation by substrate was not possible.

Specificity of the modification by DEP

DEP reacts highly specifically with histidine residues at pH 6.5–7.0 (Miles, 1977), but it has also been found to react with other nucleophiles, i.e. tyrosine, serine and residues with amino groups (Melchior and Fahrney, 1970), as well as thiols in model systems (Garrison and Himes, 1975). About 4.8 histidine residues were modified by DEP per subunit of Fe(II)-hTH1 when using a 5-molar excess of reagent (see above). Since no spectroscopic feature with maximum at about 240 nm is expected from modification of serine, lysine or arginine residues (Miles, 1977), nearly all the available DEP forms mono-*N*-carbethoxyhistidyl derivatives when it reacts with the holoenzyme. Formation

of O-carbethoxytyrosine causes a large decrease in the absorbance at 278 nm ($\varepsilon = 1310\,M^{-1}\,cm^{-1}$)(Miles, 1977), but such a spectral change was not observed for any of the three enzyme forms modified by DEP (Fig. 1 and data not shown). Modification of cysteine residues was also excluded by titration of reactive thiol groups under denaturing conditions, using Ellman's reagent (Habeeb, 1972). About seven thiol groups were measured for apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1, and this value remained unchanged following DEP treatment. Moreover, the high second-order rate constant for the inactivation of Fe(II)-hTH1 by DEP ($300\,M^{-1}\cdot min^{-1}$) indicates that this inactivation is caused by modification of histidine residues (Melchior and Fahrney, 1970; Wells, 1973; Lundblad and Noyes, 1988), although modification of lysine or arginine residues is not completely excluded.

Decarbethoxylation by hydroxylamine and recovery of the activity is currently used as an additional criterion that inactivation by DEP is due to derivatization of histidine residues with the formation of mono-N-carbethoxyhistidyl derivatives (Miles, 1977). Hydrolysis by hydroxylamine regenerates free histidine (and tyrosine), but not lysine/arginine if these amino acids have reacted with DEP (Miles, 1977). We measured a sudden decrease in ΔA_{244} to a variable extent in the different experiments (by 20–40%) following the incubation of DEP-modified apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1 with 0.01–0.1 M hydroxylamine. However, denaturation/precipitation of the enzyme occurred 2–3 min after addition of hydroxylamine. Hydroxylamine also inhibits the activity of hTH1 (approx. 45% residual activity) both in non-modified (controls) and in DEP-modified apo-hTH1 and Fe(II)-hTH1. Nevertheless, a partial recovery of the catalytic activity (approx. 11%) could be measured 10 min after addition for the DEP-modified Fe(II)-hTH1.

Apo-hTH1, Fe(II)-hTH1 and dopamine-Fe(III)-hTH1 were modified by [^{14}C]-DEP at the same conditions as in the spectroscopic studies (see above). The reaction was stopped after 20 min with 1.6 mM imidazole and the preparation and isolation of labelled tryptic peptides was performed as described (Ko et al., 1991). Due to the low specific radioactivity of the commercially available [^{14}C]-DEP (~5 mCi/mmol) and the short half-life of N-carbethoxyhistidine derivatives (55 h), the identification of the essential histidine in Fe(II)-hTH1 was not successful.

The function of the essential histidine residue

Site-directed mutagenesis studies of recombinant rat phenylalanine hydroxylase have indicated that His 284 and His 289 (corresponding to His 330 and His 335 in hTH1) are involved in iron binding (Gibbs et al., 1993). However, it is not likely that the essential histidine residue which is modified by DEP in the holoenzyme is a coordinating ligand to the catalytic iron since coordination of a histidine to the Fe(II) should protect it from DEP attack. The catalytic mechanism for the pteridine-dependent hydroxylases seems to require the activation of oxygen (Kaufman and Kaufman, 1985; Dix and Benkovic, 1988), and recent ^{1}H-NMR data support a role for the Fe(II) at the

active site in the binding, activation, and transfer of oxygen (Martínez et al., 1993a,b). One potential role for this essential histidine residue in the hydroxylation reaction is thus to function as a base catalyst promoting the activation of oxygen and its coordination to the iron in conjunction with the pteridine cofactor. Histidine residues seem to function as a base catalyst in some oxygenases containing heme iron at the active site (Erman et al., 1992) and in flavin-dependent enzymes (Lederer, 1992). Accordingly, it has recently been assigned a catalytic role to histidines 138 and 143 in phenylalanine hydroxylase from *Chromobacterium violaceum* (Balasubramaninan et al., 1994).

The essential histidine appears to become accessible to modification by DEP by a structural change accompanying the binding of Fe(II) to the apoenzyme. By Fourier transform infrared spectroscopy we have recently found that there is a change in enzyme conformation and thermal stability following the binding of Fe(II) (unpublished results). The binding of iron may also facilitate the deprotonation (formation of free base) of a nearby histidine which would then become reactive to DEP. However, rigorous proof of a location of this residue to the active site must await the elucidation of the 3-D structure of the enzyme.

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