Conformational Properties and Stability of Tyrosine Hydroxylase Studied by Infrared Spectroscopy

EFFECT OF IRON/CATECHOLAMINE BINDING AND PHOSPHORYLATION*

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The conformation and stability of recombinant tetrameric human tyrosine hydroxylase isoenzyme 1 (hTH1) was studied by infrared spectroscopy and by limited tryptic proteolysis. Its secondary structure was estimated to be 42% α-helix, 35% β-extended structures (including β-sheet), 14% β-turns, and 10% nonstructured conformations. Addition of Fe(II) or Fe(II) plus dopamine to the apoenzyme did not significantly modify its secondary structure. However, an increased thermal stability and resistance to proteolysis, as well as a decreased cooperativity in the thermal denaturation transition, was observed for the ligand-bound forms. Thus, as compared with the apoenzyme, the ligand-bound subunits of hTH1 showed a more compact tertiary structure but weaker intersubunit contacts within the protein tetramer. Phosphorylation of the apoenzyme by cyclic AMP-dependent protein kinase did not change its overall conformation but allowed on iron binding a conformational change characterized by an increase (about 10%) in α-helix and protein stability. Our results suggest that the conformational events involved in TH inhibition by catecholamines are mainly related to modifications of tertiary and quaternary structural features. However, the combined effect of iron binding and phosphorylation, which activates the enzyme, also involves modifications of the protein secondary structure.

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a non-heme iron and tetrahydrobiopterin-dependent enzyme that catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), the first and rate-limiting step in the biosynthesis of catecholamines (1, 2). The catalytic activity of TH seems to be short-term regulated by feedback inhibition by catecholamines and by reversal of this inhibition by phosphorylation (1, 3).

Until the recent cloning and overexpression of TH from different species (4–6), the source of the enzyme has mostly been the bovine adrenal medulla or pheochromocytoma (PC12) cells. However, these enzymes are isolated partially inhibited by catecholamines bound at the active site and are activated several fold by phosphorylation of Ser-40 by the catalytic subunit of cyclic AMP-dependent protein kinase (cAPK) (7–9). This activation has been related to the increased dissociation rate of catecholamines from the active site on phosphorylation (3).

Human TH exists as several different isozymes generated by alternative splicing of pre-mRNA (10, 11); isoform 1 (hTH1) is the most abundant species in the adrenal medulla and in the substantia nigra of the brain (10). When expressed in Escherichia coli, human TH isozymes are isolated as homogeneous tetrameric apoenzymes composed of identical 56-59-kDa subunits (12) that are rapidly activated (up to 40-fold) by binding of 1 atom of Fe(II) per subunit (4). The recombinant human enzymes are inhibited by catecholamines that chelate iron at the active site, and this inhibition is partially reversed by phosphorylation of Ser-40 by cAPK (3, 12, 13).

The three-dimensional structure of TH remains to be determined, and the potential conformational changes associated with the activation by phosphorylation and inhibition by catecholamines are not characterized. It has been postulated that phosphorylation induces a conformational change that decreases the thermositivity of the enzyme (14, 15). However, the influence of the enzyme-bound catecholamines on these effects was not considered. In the present work, we have studied the secondary structure and stability of recombinant hTH1, with special reference to the effects of its activation by Fe(II) and phosphorylation by cAPK and its inhibition by dopamine. As experimental techniques, we have used infrared (IR) spectroscopy and limited tryptic proteolysis.

MATERIALS AND METHODS

Dopamine and deuterium oxide were from Sigma. Human TH isoform 1 (hTH1) was expressed in E. coli and purified to homogeneity as described (4, 12). Buffers were passed through a Chelex-100 ion-exchange resin to avoid iron contamination. The enzyme preparations used in this study contained 0.02 ± 0.01 atoms of iron/subunit, as determined by atomic absorption spectrometry, and was considered to represent the apoenzyme (apo-hTH1). The holoenzyme, Fe(II)-hTH1, was prepared by incubation of apo-hTH1 with equimolar concentrations of ferrous ammonium sulfate for 5 min at 20 °C (4, 16), and the dopa
mime Fe(II)-hTH1 complex was obtained by the incubation of Fe(II)-
hTH1 with equimolar concentrations of dopamine (16). The concentra
tion of purified recombinant enzyme was determined by the absorbance at 280 nm (ε280 = 10.4 cm M−1) at neutral pH (7). The catalytic subunit of cAPK was purified from bovine heart as described (17). Phosphorylated Fe(II)-hTH1 at Ser-40 was prepared from the phosphorylated apo-hTH1 as described for the nonphosphorylated form of the enzyme (see above).

Phosphorylation of hTH1—apo-hTH1 (25 μM) was incubated with 0.1 mg/ml of bovine heart cAPK catalytic subunit, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 mM MgCl2, 2 mM ATP, 5 M
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**Fig. 1.** IR spectra of recombinant human tyrosine hydroxylase (hTH1) in D$_2$O buffer: Apo-hTH1 (continuous line) and dopamine-Fe(III)-hTH1 (broken line). A, original spectra in the 1800–1500 cm$^{-1}$ region; B, deconvoluted spectra in the amide I region. Fourier self-deconvolution was performed using a Lorentzian line shape of 18 cm$^{-1}$ half-width and a resolution-enhancement factor of 3.

[Scans and diagrams of spectra]

**Fig. 2.** Amide I band decomposition of nonphosphorylated (A) and phosphorylated Fe(II)-hTH1 (B) in D$_2$O buffer. The values obtained for decomposition parameters are shown in Table I.

**RESULTS**

Secondary Structure of Recombinant Human Tyrosine Hydroxylase (hTH1)—Fig. 1 shows the 1800–1500 cm$^{-1}$ region of the original and the amide I band of the deconvoluted infrared spectra of apo-hTH1 and the dopamine-Fe(III)-hTH1 complex, recorded in D$_2$O medium. The deconvoluted spectra of these samples revealed that the amide I band can be resolved into several components both in D$_2$O (Fig. 1) and H$_2$O (data not shown). Assignment of individual band components to specific types of secondary structure has been discussed recently (22).

The intense and well resolved component bands appearing at around 1654 and 1635 cm$^{-1}$ have been assigned to $\alpha$-helical and $\beta$-sheet conformations, respectively. However, nonstructured conformations may also contribute to the intensity of the helical and $\beta$-sheet components in H$_2$O and D$_2$O, respectively (see below). Minor components are seen in both solvents at around 1668 and 1677 cm$^{-1}$, which mainly arise from $\beta$-turns. The rather weak component band at around 1625 cm$^{-1}$ has been assigned to $\beta$-strands, extended chains not forming $\beta$-sheets, and have been related, in native oligomeric proteins, to protein-protein contacts (21, 23, 24).

Quantification of protein secondary structure was performed following a procedure described recently (21, 23). Fig. 2 illustrates the results obtained from the band-fitting analysis of the original amide I band corresponding to nonphosphorylated and phosphorylated Fe(II)-hTH1. Measurements in H$_2$O and D$_2$O improve the assignment of the band components due to the...
isotopic shift that is known to occur in certain band components when transferred to the latter medium (23). The values obtained for the band position and percentage area of each component for apo-hTH1 and Fe(II)-hTH1 are presented in Table I. The difference between the spectra recorded in H2O and D2O in percentage area of the band around 1655 cm\(^{-1}\) was 8–10%, but a defined band did not appear at 1640 cm\(^{-1}\) in D2O, as expected from a shift of the band corresponding to unordered structures with backbone deuteration. Instead, the area of the band attributable to \(\beta\)-sheet structure, which appears at 1636 cm\(^{-1}\) (25), increased, and its position shifted upward upon isotopic substitution (see band positions in Table I), indicating that the bands corresponding to \(\beta\) and nonregular structure overlap in D2O.

The effect of phosphorylation at Ser-40 by cAPK on the secondary structure of Fe(II)-hTH1 is also shown in Table I. The spectra of the phosphorylated apo-hTH1, Fe(II)-hTH1, and Fe(II)-hTH1 in the presence of equimolar amounts of dopamine look very similar to those described above for the nonphosphorylated samples, including the number and position of the amide I component bands (data not shown). It should be noted that binding of dopamine to the phosphorylated Fe(II)-hTH1 is almost abolished due to a dramatic decrease in the binding affinity (13). A quantitative estimate of the secondary structure of nonphosphorylated and phosphorylated apo-hTH1 and their ligand-bound forms is given in Table II. No significant differences (>3%) were detected between the nonphosphorylated and phosphorylated forms of apo-hTH1. By contrast, the addition of an equimolar concentration of Fe(II), which generates the catalytically active enzyme form, induced a conformational change in phosphorylated apo-hTH1, notably an increase of about 10% in the \(\alpha\)-helix content, concomitant with a loss of unordered structure. These changes are maintained in the presence of equimolar concentrations of dopamine.

Effect of Ligand Binding on the Thermal Stability of hTH1—Further insight into the structural changes that occur on ligand binding and phosphorylation of hTH1 was obtained from thermal stability studies. The deconvoluted IR spectra of apo-hTH1 in D2O buffer revealed major changes in the amide I (1700–1610 cm\(^{-1}\)) and amide II (1550 cm\(^{-1}\)) modes between 44 and 52°C (Fig. 3). These changes included a broadening of the overall amide I contour and the appearance of well defined components at 1618 and 1685 cm\(^{-1}\), highly characteristic of thermally denatured proteins (26), which represent extended structures formed on aggregation of the unfolded proteins (21, 27). Loss of the residual amide II band at around 1550 cm\(^{-1}\), within the above mentioned temperature interval, indicated that thermal denaturation exposed the protein core to deuterium exchange.

Thermal unfolding of the enzyme was followed by using an

### Table I

| Band position \(\%\) area | \(\alpha\)-helix | \(\beta\)-sheet | \(\beta\)-turns | Unordered | \(\beta\)-strand |
|--------------------------|-----------------|----------------|--------------|-----------|-------------|
| \(H_2O\)                  | 42              | 30             | 14           | 10        | 5           |
| \(D_2O\)                  | 43              | 33             | 14           | 8         | 2           |
| \(\alpha\)-helix          | 46              | 30             | 11           | 12        | 2           |
| \(\beta\)-sheet           |                |                |              |           |             |
| \(\beta\)-turns           |                |                |              |           |             |
| Unordered                 |                |                |              |           |             |
| \(\beta\)-strand          |                |                |              |           |             |

### Table II

| Structure | Nonphosphorylated | Phosphorylated |
|-----------|-------------------|----------------|
| apo-hTH1  | Fe(II)-hTH1       | dopamine + Fe(II)-hTH1 |
| \(\alpha\)-helix | 42 | 43 | 46 |
| \(\beta\)-sheet  | 30 | 33 | 30 |
| \(\beta\)-turns  | 14 | 14 | 11 |
| Unordered     | 10 | 8  | 12 |
| \(\beta\)-strand | 5  | 2  | 6  |

*The values are rounded off to the nearest integer.*
empirical parameter, defined as the intensity ratio of the amide I band at 1618 cm\(^{-1}\) to that at 1650 cm\(^{-1}\) (I\(_{1618}\): I\(_{1650}\)) as a function of temperature for apo-hTH1 (*), Fe(II)-hTH1 (●), and Fe(II)-hTH1 in the presence of equimolar amounts (○) or a 50 molar excess of dopamine (△).

![Figure 4. Thermal stability of nonphosphorylated (A) and phosphorylated (B) hTH1. Ratio of amide I band intensity at 1618 to that at 1650 cm\(^{-1}\) (I\(_{1618}\): I\(_{1650}\)) as a function of temperature for apo-hTH1 (*), Fe(II)-hTH1 (●), and Fe(II)-hTH1 in the presence of equimolar amounts (○) or a 50 molar excess of dopamine (△).](image)

**DISCUSSION**

We have studied the secondary structure of human tyrosine hydroxylase (hTH1) and the conformational changes related to phosphorylation on the global conformation of hTH1 was also examined by studying the susceptibility of the different enzyme forms to limited tryptic proteolysis. Such treatment of rat and bovine TH produces a 34-kDa core fragment with an increased catalytic activity (1, 28). As expected, the activity of hTH1 also increased when apo-hTH1 was incubated for 20 min at 25 °C with trypsin, with maximal activation (about 40%) at a trypsin:protein ratio of 0.005:1 (µg:µg). However, as shown by SDS-polyacrylamide gel electrophoresis (Fig. 5), the major product of the nonphosphorylated apo-hTH1 was a 47-kDa protein fragment. The same species were observed with the ligand-bound forms of the enzyme, regardless of its phosphorylation state. At higher protease concentrations, the 47-kDa fragment was further digested to species of lower electrophoretic mobility, and extensive proteolysis was observed at a trypsin:protein molar ratio of 0.1:1 (Fig. 5). Reconstitution of the nonphosphorylated (Fig. 6A) and phosphorylated apo-hTH1 (Fig. 6B) with Fe(II) increased the resistance to proteolysis of the active 47-kDa enzyme form. A comparison of the fragmentation patterns corresponding to phosphorylated and nonphosphorylated holoprotein revealed that the 47-kDa fragment of the former was more resistant to proteolysis, in good agreement with the thermal stability studies. Dopamine binding to nonphosphorylated holoprotein further stabilized the 47-kDa species (Fig. 6A), whereas no effect on the degradation pattern was found on addition of an equimolar amount of dopamine to the phosphorylated holoenzyme prior to proteolysis (data not shown).

![Figure 5. SDS-polyacrylamide gel electrophoresis showing the tryptic digestion of apo-hTH1.](image)

**Table III**

Parameters for the thermal unfolding of nonphosphorylated and phosphorylated apo-hTH1, Fe(II)-hTH1, and Fe(II)-hTH1 plus dopamine

|                | Non-Phosphorylated | Phosphorylated |
|----------------|--------------------|----------------|
| **T\(_m\) (°C)** | ΔT (°C) | **T\(_m\) (°C)** | ΔT (°C) |
| hTH1           | 49               | 8              | 49               | 9              |
| Fe(II)-hTH1    | 54               | 12             | 57               | 17             |
| Fe(II)-hTH1:dopamine (1:1) | 62               | 20             | 57               | 17             |
| Fe(II)-hTH1:dopamine (1:50) | 61               | 19             | 50               | 10             |

\(T\(_m\), \) midpoint denaturation temperature; ΔT, temperature interval in which thermal denaturation takes place.
its activation by Fe(II) and phosphorylation of Ser-40 as well as its inhibition by dopamine, using the conformational sensitivity of the infrared amide I band and the susceptibility to limited proteolysis.

Structural Changes Related to hTH1 Activation by Fe(II) and Inhibition by Catecholamines—No significant changes were observed in the secondary structure of apo-hTH1 when reconstituted with Fe(II); the major effect was an increase in protein stability. Thus, our results support a role for iron in the stabilization of TH conformation, in addition to its catalytic function. The only remarkable difference detected in the IR spectrum of the protein on dopamine binding is the loss of the 1625 cm$^{-1}$ band component. A band at this position has been associated with intersubunit contacts, and it has been described in dimeric (21) but not in monomeric (30) cytochrome c oxidase. Therefore, its disappearance may indicate a catecholamine-mediated weakening of intersubunit interactions within the protein tetramer, as also suggested by the increase in the width of the thermal unfolding transition. A broad transition would indicate a more or less independent subunit unfolding, whereas a sharp transition would suggest that the tetramer behaves essentially as a cooperative unit (31). However, the stabilizing effect of iron on the native conformation of hTH1 (see above) is further strengthened on dopamine binding to the holoenzyme. This effect is most likely on the monomer of the tetrameric enzyme, since each subunit binds equimolar amounts of the ligands, as found with other soluble proteins (24, 31, 32). Thus, inhibition of the enzyme by catecholamines is associated with a stabilization of its conformation and could be related to the proposed existence of two forms of the bovine enzyme (33), i.e. a labile active form and a stable inactive form, generated by catecholamine binding to the labile form.

Effect of Phosphorylation on the Conformation of hTH1—The molecular and/or structural mechanism by which phosphorylation of Ser-40 results in an activation of TH, kinetically represented by a decrease in the apparent $K_m$ for the cofactor and an increase in $K_i$ for dopamine, is yet unknown (1, 12, 13). Although phosphorylation of apo-hTH1 does not significantly modify its conformation, a significant finding in this work is that the secondary structure of the catalytically active holoenzyme does indeed depend on its phosphorylation state. When phosphorylated apo-hTH1 is reconstituted with iron, a significant (~10%) increase in the $\alpha$-helical content and a concomitant decrease in unordered structure were observed. The fact that this structural transition is triggered by iron binding suggests a close proximity and/or interaction of the phosphorylation site (Ser-40 at the regulatory N-terminal domain) and the iron binding site (at the catalytic core domain). A recent study based on circular dichroism has provided similar values (~50%) for the $\alpha$-helix content of TH isolated from rat PC12 cells (15) as those found in this work for hTH. However, our results differ from their proposal that phosphorylation of TH destabilizes its conformation. The phosphorylated active holoenzyme (Fe(II)-hTH1) also shows a slightly increased thermal stability (Table III) and resistance to limited proteolysis (Fig. 6) as compared with the nonphosphorylated form. This apparent discrepancy may be due to either (i) different enzyme sources and/or (ii) TH isolated from rat PC12 cells contains catecholamines bound at the active site (9) and, therefore, the decreased thermostability could be a consequence of a phosphorylation-induced catecholamine release from the active site.

Proteins that undergo regulatory serine phosphorylation may be classified into two groups, i.e. those that undergo a global conformational change upon phosphorylation and those that do not (34). An example of the first group is glycogen phosphorylase whose N-terminal segment, bearing the phosphorylation site (Ser-14), becomes helical upon phosphorylation and those that do not (34).

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