Thermal Stability and CD Analysis of Rat Tyrosine Hydroxylase†

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ABSTRACT: Tyrosine hydroxylase is the rate-limiting enzyme of catecholamine biosynthesis. It is a homotetramer made up of 56 kDa subunits. We examined the thermal stability of tyrosine hydroxylase purified from a rat pheochromocytoma cell line and investigated the relationship between enzyme activity and stability. Thermal stability was assessed by incubating the enzyme at an elevated temperature. Unfolding of the protein was followed by measuring the loss of circular dichroism (CD) at 220 nm. The CD loss was biphasic, with half-lives of 2 and 14 min at 55 °C in 100 mM potassium phosphate, pH 6.0. The rate of loss of enzyme activity paralleled the longer half-life under these conditions. This indicates that the structure of the active site is not appreciably changed by the unfolding events corresponding to the first phase. Moreover, unfolding as assessed by the CD spectrum and activity was not reversible and did not exhibit a well-defined midpoint temperature or T_m. The thermal stability of the enzyme was altered by several factors that influence activity. The enzyme at pH 6.0 was less stable ($t_{1/2}$ = 6.2 and 29 min) than the enzyme at pH 7.2 (a single $t_{1/2}$ of 64 min). Phosphorylated tyrosine hydroxylase had shorter half-lives ($t_{1/2}$ of 2 and 16 min) than the nonphosphorylated enzyme ($t_{1/2}$ 6.2 and 29 min) at pH 6.0, 50 °C, in 100 mM phosphate. Moderate changes in phosphate concentration had dramatic effects on enzyme stability. Decreasing the phosphate concentration from 50 to 10 mM (pH 6.0) increased the half-life from 2 and 23 min to greater than 120 min. Phosphorylation, decreased pH, or increased buffer activate tyrosine hydroxylase and produce a less stable protein. This contrasts to the responses to heparin and DNA. These polyanions activate tyrosine hydroxylase, but they increased thermal stability. These polyanions may bind across adjacent protein domains and thereby decrease the rate of unfolding.

Tyrosine hydroxylase (EC 1.14.16.2) is the rate-limiting enzyme in catecholamine biosynthesis in vivo (Levitt et al., 1965). It catalyzes the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (DOPA). The rat enzyme is a homotetramer (Okuno & Fujisawa, 1982), with a subunit size of 60 000 daltons (as determined by SDS−PAGE) and a holoenzyme size of approximately 260 000 daltons as determined by gel filtration (Okuno & Fujisawa, 1982). It is an iron-containing enzyme, and iron is essential for enzyme activity (Hoeldtke & Kaufman, 1977; Dix et al., 1987; Fitzpatrick, 1989). Rat cDNA encodes a protein of 498 amino acids (Grima et al., 1985), including an amino-terminal methionine which is subsequently cleaved from the protein (Fujisawa & Okuno, 1987). The final product has a calculated molecular mass of 55 903 daltons, less than the 60 000 daltons estimated by SDS−PAGE (Okuno & Fujisawa, 1982).

Tyrosine hydroxylase activity is altered in vitro by many factors, including pH, ionic strength, and polyanions such as heparin and nucleic acids (Kuczenski & Mandell, 1972; Katz et al., 1976; Nelson & Kaufman, 1987; Gahn & Roskoski, 1993). The enzyme is phosphorylated and activated by several kinases, including protein kinase A (cyclic AMP-dependent protein kinase) (Joh et al., 1978; Vulliet et al., 1980).

Lazar and co-workers (Lazar et al., 1981) assessed tyrosine hydroxylase thermal stability by measuring the rate of loss of activity during incubation at an elevated temperature (50 °C). They noted that the half-life of phosphorylated tyrosine hydroxylase activity at 50 °C decreases to one-third of that of the nonphosphorylated enzyme (5 min versus 15 min). Lazar et al. (1981) postulated that the covalent modification of tyrosine hydroxylase by phosphorylation induces a conformational change that decreases thermostability.

The purpose of this research was to examine the thermal stability of tyrosine hydroxylase and to determine the relationship between enzyme activity and thermal stability. We found that activated tyrosine hydroxylase was generally less stable than the nonactivated enzyme. The effects of polyanions (heparin and DNA), however, differed. These agents increased enzyme activity and stabilized the enzyme. Tyrosine hydroxylase, a complex regulatory enzyme (Zigmond et al., 1989), failed to exhibit a well-defined midpoint transition temperature as assessed by the circular dichroism (CD)[1] spectrum. Moreover, unfolding was irreversible.

MATERIALS AND METHODS

Materials. Tyrosine hydroxylase was purified by the method of Gahn and Roskoski (1991) from rat PC12 cells. The catalytic subunit of protein kinase A was purified by the method of Hartl and Roskoski (1982). 6(*R*)-5,6,7,8-Tetrahydro-L-biopterin was purchased from Dr. B. Schircks (Jona, Switzerland). L-[3,5-^{3}H]Tyrosine was from NEN

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[1] Abbreviations: CD, circular dichroism; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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Research Products (Boston, MA). Salmon sperm DNA, heparin, and buffers were from Sigma Chemical Co. (St. Louis, MO).

CD Measurements. CD was measured with a JASCO J720 spectropolarimeter calibrated with ammonium *d*-10-camphorsulfonate according to JASCO technical specifications. A 1 mm path-length cell was used, and the sample chamber was flushed continuously with N_2. Tyrosine hydroxylase (500 μg/mL) was incubated in potassium phosphate at the indicated pH and temperature. To measure the CD spectrum of tyrosine hydroxylase, samples were scanned 10 times, from 185 to 250 nm, in 0.1 nm increments, 200 nm/min, and averaged. The no-enzyme spectrum was subtracted, and noise reduction calculations were performed using the J720 spectrum processing software. The time-dependent CD signal was monitored at 220 nm. The enzyme solution was placed in a water-jacketed cell with continuous water circulation. Water was maintained at the indicated temperature with a Neslab Endocal circulating water bath. Measurements were initiated about 20 s after the sample was exposed to the higher temperature. CD measurements at 220 nm were recorded at 5 s intervals until there was no further change in the CD signal. The CD cell was cleaned with concentrated chromic acid between each determination to remove any tyrosine hydroxylase adhering to the inner surface. To prevent bubble formation during the experiment, buffer was first heated to near-boiling and then cooled to room temperature. Protein was added to the buffer, and the experiment was initiated immediately.

Secondary structure estimations were computed with the JASCO SSE-338 program that compares the actual protein CD spectrum (from 190 to 240 nm) with reference spectra for four conformations (α-helix, β-sheet, β-turn, and random or unordered). These spectra are based on the CD spectra of 15 reference proteins with known secondary and tertiary structure [data from Table III of Yang et al. (1986)]. In this program, the calculations are constrained such that the sum of the percent of each type of structure equals 100%. The half-lives for the loss of CD signal were calculated with the Marquardt–Feldberg nonlinear least-squares fitting routine (Bevington, 1969) used in the program FIT-87, kindly provided by Dr. Christopher J. Batie (Louisiana State University Medical Center). It assumes that each experimental curve is composed of one or more exponential decay curves. This program can analyze a maximum of 400 data points, while considerably more data were collected. The CD signal fluctuated for the first minute of the incubation, and these data were excluded from half-life calculations. Since the CD changed more rapidly in the earlier part of each experiment, we used a value every 5 s for 8 min, every 10 s for the next 16 min, every 20 s for the next 32 min, and every 40 s thereafter. All of the thermal unfolding figures express edited data.

RESULTS

CD and Activity. Figure 1 shows the CD spectrum of native tyrosine hydroxylase at pH 6.0. Secondary structure calculations indicate 55% α-helix and 22% β-sheet for the native protein. The CD spectrum was similar under a variety of conditions that altered activity, including different pH values, state of phosphorylation, and different salt concentrations (not shown). At pH 6.0 after incubation at 65 °C for

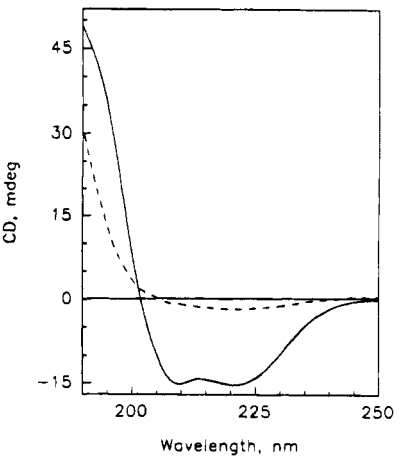

FIGURE 1: CD spectrum of tyrosine hydroxylase before or after incubation at 65 °C. The spectrum of 500 μg/mL tyrosine hydroxylase in 20 mM potassium phosphate, pH 6.0, was measured before (—) or after (- - -) incubation at 65 °C for 80 min.

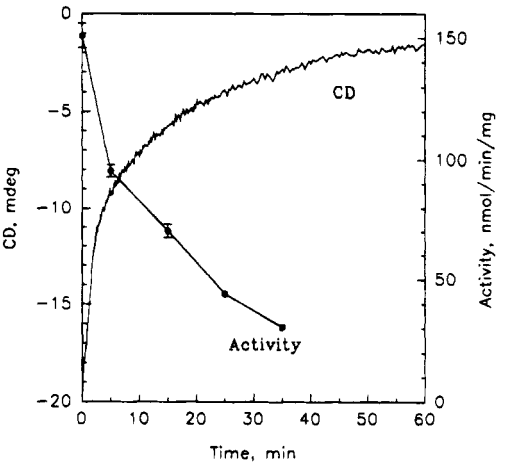

FIGURE 2: Tyrosine hydroxylase CD (220 nm) and activity. Enzyme was incubated in 100 mM potassium phosphate, pH 6.0, at 55 °C, and CD was followed over time. In a parallel sample, aliquots were removed, and activity was assessed as described in text.

80 min, the CD spectrum was greatly altered, with almost no signal at 220 nm. The secondary structure calculations based upon the CD spectrum obtained after heating did not yield a good fit, suggesting that the heated protein possessed an ill-defined secondary structure. Moreover, the sample lost all enzymatic activity (data not shown). At 220 nm, both α-helix and β-strand structures give a negative CD signal. The loss of either type of structure results in the loss of this negative signal, and CD at 220 nm monitors the loss of both α-helix and β-strand structure during thermally-induced unfolding. Most buffers and oxygen do not interfere with measurements at 220 nm, allowing more freedom to manipulate the experimental system. The loss of the CD signal and activity was irreversible.

At 55 °C, in 100 mM potassium phosphate (pH 6.0), the negative CD signal at 220 nm was gradually lost, with no further change observed after about 2 h (Figure 2). At 55 °C, the loss of CD best fit a biphasic curve composed of two exponential portions, with half-lives of 2 and 14 min. These data were obtained when tyrosine hydroxylase was incubated at a single elevated temperature. This protein failed to exhibit a well-defined T_m when spectra were taken as the temperature was gradually increased. We therefore

Table 1: Half-Lives of Tyrosine Hydroxylase CD Loss at Elevated Temperature[a]

pH	temperature (°C)		$t_{1/2}$ (min)
6.0	70		0.2, 8.5
	60		1.0, 13
	50		3.2, 31
6.0	50	phosphorylated	1.7, 16
		nonphosphorylated	6.2, 29
7.2	50	phosphorylated	11, 88
		nonphosphorylated	64

[a] The CD signal (220 nm) of tyrosine hydroxylase in 100 mM potassium phosphate was followed until no further change in CD was observed. Half-lives of CD loss were calculated as described in the text. Half-lives less than 2 min have an error of ±0.5 min.

measured the CD spectra of tyrosine hydroxylase at a single temperature as a function of time to assess the stability of the protein. The temperature was changed in our experiments to accommodate different rates of unfolding, depending upon the experimental conditions.

We compared the loss of tyrosine hydroxylase activity with the loss of CD in the preceding experiment (55 °C, 100 mM potassium phosphate, pH 6.0). An identical solution was placed in the CD cell and incubated at 55 °C. At the times indicated, aliquots (10 μL) of the solution were removed and diluted in 190 μL of ice-cold PIPES buffer, pH 6.0. Activity was assessed immediately by the method of Reinhard et al. (1986) with modifications (Gahn & Roskoski, 1993), in 50 mM PIPES (pH 6.0), 100 μM tyrosine, and 1 mM tetrahydrobiopterin at 37 °C for 10 min. A time-dependent loss of activity was observed (Figure 2). The activity loss fit a single-phase exponential curve, with a half-life of 15 min. This half-life was experimentally indistinguishable from the second half-life of the loss of CD signal (Table 1). The data indicate that the catalytic site was destroyed during the second phase of thermal unfolding under these conditions. Increasing the temperature from 50 °C to 60 °C or 70 °C increased the rate of CD loss (Table 1). The half-lives for both phases of CD loss decreased as the temperature was increased.

pH Effects on Tyrosine Hydroxylase Thermal Stability. Tyrosine hydroxylase activity is pH-dependent and is more active at pH 6.0 than at pH 7.2 (Richtand et al., 1985). We compared the loss of the CD signal at pH 6.0 and 7.2 (100 mM potassium phosphate, 50 °C). CD loss proceeded at a distinctly slower rate at pH 7.2, with an increase in both half-lives (Table 1). The rate of CD loss at pH 7.2 was slower than that at pH 6.0 at higher and lower temperatures (not shown). This suggests that there are structural differences in the enzyme at pH 6.0 and 7.2 that affect both activity and thermal stability.

Stability of Phosphorylated Tyrosine Hydroxylase. Protein kinase A phosphorylation increases tyrosine hydroxylase activity. At pH 6.0 (50 °C), phosphorylated tyrosine hydroxylase exhibited a 75% decrease in the first half-life and a 50% decrease in the second half-life (Table 1). Nonphosphorylated enzyme was also more stable at pH 7.2 than the phosphorylated enzyme. These results are consistent with those of Lazar and co-workers (Lazar et al., 1981), who found that phosphorylation decreased the thermal stability of tyrosine hydroxylase (as determined by enzyme activity). They calculated a half-life of 15 min for the unphosphorylated enzyme and 5 min for the phosphorylated enzyme.

Table 2: Half-Lives of Tyrosine Hydroxylase CD Loss in Potassium Phosphate and KCl[a]

salt (mM)	pH	temperature (°C)	$t_{1/2}$ (min)
potassium			
150	6.0	55	1.8, 23
50			1.8, 23
25			6.2, 26
10			>120
150	7.2	65	0.7, 48
50			1.4, 104
25			>120
KCl			
180	6.0	50	2.6, 31
80			2.7, 41
0			13, 72
130	7.2	70	0.9, 7.5
80			1.5, 10.3
50			3.2, 72
0			>120

[a] The CD signal (220 nm) of tyrosine hydroxylase in varying amounts of potassium phosphate or in 20 mM potassium phosphate with varying KCl was followed until no further change in CD was observed. Half-lives of CD loss were calculated as described in the text. Half-lives less than 2 min have an error of ±0.5 min.

These half-lives of activity loss cannot, unfortunately, be compared directly to the half-lives determined here by CD changes because Lazar and co-workers (Lazar et al., 1981) did not specify their experimental conditions (pH, buffer, or salt). In the experiments reported here, the thermal stability of the enzyme was dependent on these factors.

Salt Effects on Tyrosine Hydroxylase Thermal Stability. Tyrosine hydroxylase is activated by salts (Katz et al., 1976), and this prompted us to investigate the stability of tyrosine hydroxylase as a function of this parameter. At pH 6.0 (55 °C), the tyrosine hydroxylase exponential rates of CD signal loss decreased quickly in the presence of 50−150 mM potassium phosphate. Decreasing the phosphate concentration to 25 mM markedly slowed the rates of CD loss, and further decreasing the phosphate concentration to 10 mM almost completely prevented the CD loss over the length of the experiment (Table 2). At pH 7.2 (65 °C), similar results were obtained (Table 2). The CD signal decreased quickly in 150 mM potassium phosphate, slowly in 50 mM phosphate, and very little in 25 mM phosphate over the length of the experiment (100 min). We also incubated the enzyme in 20 mM potassium phosphate and altered the ionic strength by adding KCl instead of potassium phosphate. Under these conditions at both pH 6.0 and 7.2, the enzyme in solutions with a higher KCl concentration exhibited an increased rate of loss of the CD signal (Table 2).

Polyanion Effects on Stability. In all of the above experiments, more active enzyme was less stable than less active enzyme. In contrast to these factors, stability changes produced by activation with polyanions show an opposite pattern. Heparin or DNA, which activates tyrosine hydroxylase, decreased the rate of CD loss at pH 6.0, in 50 mM potassium phosphate (Figure 3, Table 3), indicating increased thermal stability in the presence of polyanions. Heparin was effective at lower concentrations than DNA, which parallels their relative effectiveness in activating the enzyme (Gahn & Roskoski, 1993). At pH 7.2, where polyanions affected activity less, DNA had less effect on CD loss as well (Table 3).

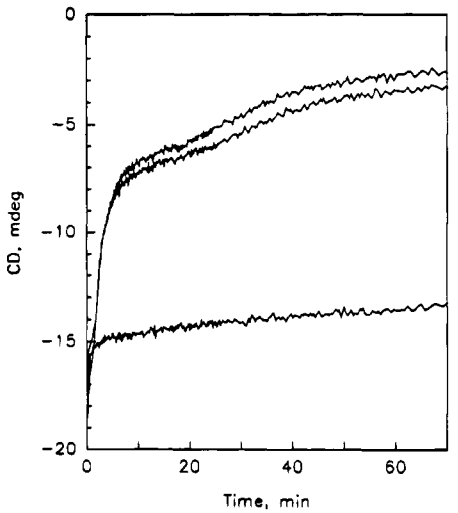

FIGURE 3: Dichroic analysis of tyrosine hydroxylase with heparin. The CD (220 nm) was followed in 50 mM potassium phosphate, pH 6.0, at 60 °C in the presence of (top to bottom) 0, 1, or 10 μg/mL heparin.

Table 3: Half-Lives of Tyrosine Hydroxylase CD Loss with Polyanions[a]

pH	temperature (°C)	polyanion	$t_{1/2}$ (min)
6.0	60	none	2.1, 13
		150 μg/mL DNA	2.6, >60
		300 μg/mL DNA	>60
6.0	60	none	1.8, 14
		1 μg/mL heparin	2.0, 13
		10 μg/mL heparin	>60
7.2	75	none	1.2, 89
		300 μg/mL DNA	2.8, >120
		1000 μg/mL DNA	2.4, >120

[a] The CD signal (220 nm) of tyrosine hydroxylase in 50 mM potassium phosphate was followed until no further change in CD was observed. Half-lives of CD loss were calculated as described in the text. Half-lives less than 2 min have an error of ±0.5 min.

DISCUSSION

Lazar and co-workers (Lazar et al., 1981) showed, by following the loss of tyrosine hydroxylase activity at elevated temperature, that phosphorylation of the enzyme decreased its thermal stability. Using circular dichroism to follow unfolding of tyrosine hydroxylase, all factors that changed activity were found to alter the thermal stability of tyrosine hydroxylase as well.

Several small proteins such as ribonuclease T1 display thermal unfolding curves with a well-defined T_m (Pace et al., 1990). The thermal unfolding of ribonuclease T1 is reversible, and the T_m can be approached from lower or higher temperatures. There are few studies on the thermal denaturation of multimeric regulatory enzymes such as tyrosine hydroxylase because of their more intricate behavior. More complex unfolding curves are frequently observed with multidomain proteins where domains unfold independently (Pace et al., 1990). The CD analysis indicates that tyrosine hydroxylase unfolded irreversibly and did not exhibit a well-defined T_m. Because of the irreversible (nonequilibrium) nature of unfolding, we were unable to calculate a standard free energy difference between the folded and unfolded states using the equilibrium constant. We determined the half-lives for unfolding at a given temperature under specific conditions and determined the influence on these conditions on thermal stability. This technique promises to be of general utility in the study of protein unfolding, since it does not rely on reversible unfolding.

Chen et al. (1990) measured the time-dependent unfolding of angiotensin converting enzyme produced by guanidinium chloride. They found that this monomeric enzyme (130 kDa) has an unfolding pattern with no well-defined transition from the folded to the unfolded state. They found that the activity loss occurs at a concentration of guanidinium chloride that fails to produce a change in CD (or intrinsic fluorescence). In addition, they measured the rate of change in the presence of a given concentration of guanidinium chloride and found that activity loss occurs in two phases. CD loss coincides with the slower phase, and fluorescence loss occurs at an intermediate rate. On the basis of these experiments, the authors suggest that the active site of angiotensin converting enzyme is less stable than the remainder of the protein. The active site of ribonuclease A is also less stable than the remainder of the protein to guanidine denaturation (Liu & Tsou, 1987). In contrast to both of these enzymes, the loss of tyrosine hydroxylase activity coincided with the slower phase of thermal unfolding at 55 °C, suggesting that the active site is more stable than other parts of the enzyme.

The far-UV CD spectrum of tyrosine hydroxylase was unchanged following phosphorylation by protein kinase A. Extrinsic fluorescence does decrease, however, providing evidence for an alteration in the tertiary structure (Gahn & Roskoski, 1993). We observed, moreover, that phosphorylated tyrosine hydroxylase exhibited decreased thermal stability.

A decrease in pH from 7.2 to 6.0 activates the enzyme and decreased its stability. The predominant amino acid whose ionization state changes in this pH range is histidine. Our results suggest that protonation of histidines destabilized tyrosine hydroxylase. An increase in salt concentration decreased its stability. This is unusual because proteins are usually stabilized by high ionic strength (Timasheff & Arakawa, 1990). However, studies of salt effects on protein stability are usually performed with 500 mM salt and greater, and not concentrations between 10 and 150 mM as were used here. Destabilization by salts suggests that thermal unfolding was accompanied by the disruption of ionic bonds.

Polyanions, which had effects opposite to pH, phosphorylation, or ionic strength, increased the thermal stability and activity of tyrosine hydroxylase. Heparin binds to acidic and basic fibroblast growth factors (Burgess & Maciag, 1989). Heparin protects these growth factors from heat inactivation. Incubating the growth factors at elevated temperature (65 °C for 5 min) results in the loss of biological activity, but heparin (10 μg/mL) protects against inactivation (Gospodarowicz & Cheng, 1986). Heparin also protects the growth factors from proteolytic degradation by trypsin or thermolysin in vitro or by proteases in culture (Rosengart et al., 1988; Lobb, 1988; Sommer & Rifkin, 1989). Heparin is thought to bind to fibroblast growth factors over a large area rather than at a single site (Burgess & Maciag, 1989). We suggest that polyanions such as heparin or DNA bind to the surface of tyrosine hydroxylase, perhaps across subunits or domains, rendering the protein less susceptible to thermal unfolding.

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