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The Effect of Temperature on Mixed Function Amine Oxidase Intrinsic Fluorescence and Oxidative Activity

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SUMMARY

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Using temperature as a perturbant, we have shown that mixed function amine oxidase undergoes structural changes *in vitro* which are correlated with changes in oxidative activity toward methimazole and N,N-diethylaniline. The observed changes between 30°-40° are irreversible, as shown by fluorescence, circular dichroism, and kinetic measurements. Although these studies show that the enzyme is remarkably sensitive to heat, *in vivo* properties may be different owing to possible endogenous ligands and lipid environment. The techniques used are sensitive and have potential for selective monitoring of mixed function amine oxidase properties in biological systems.

INTRODUCTION

Prior to 1962, cytochrome P-450 was generally considered the enzyme solely responsible for the biological oxidation of nitrogen in organic compounds (1). However, in 1962, Baker and Chaykin (2) published a report suggesting that trimethylamine may be N-oxidized by an enzyme other than cytochrome P-450. By 1974, Ziegler and coworkers (3-7) and Uehleke *et al.* (8) demonstrated that a flavoprotein is capable of carrying out mixed function oxidation of amines and sulfur-containing compounds. In an attempt to classify N-oxidation reactions, Gorrod (9) reviewed the literature, and after considering a number of parameters including substrate stereochemistry, established a simple three category classification for nitrogenous substrates: I; basic amines ($pK_a \geq 8$)[3] oxidized solely by mixed function amine oxidase, II; substrates containing nonbasic nitrogen ($pK_a \leq 1$) N-oxidized by cytochrome P-450, and III; compounds with pK_as between 1 and 7 that are substrates for both enzymes. Unfortunately, there are so many exceptions to this latter group that specific enzymatic classification based on pK_a is of little value. For example, aniline is metabolized only by cytochrome P-450 (10), while N-methylaniline, N,N-dimethylaniline, and 4-acetylaminobiphenyl are metabolized only by mixed function amine oxidase (3, 11-13), although all have pK_as between 3-5.2. Thus, it is evident that factors other than pK_a are important in defining substrate-oxidase activity. In fact, recent work in our laboratory

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[3] The abbreviations used are: pK_a, HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CD, circular dichroism;

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has indicated that sterically hindered amines with $pK_a s \geq 8$ are not N-oxidized by mixed function amine oxidase,[4] suggesting that steric hindrance is a factor.

The importance of N-oxidation as a metabolic route for drugs in humans has been largely overlooked: studies concentrated more on oxidation of carbon adjacent to nitrogen. Recently, Gold and Ziegler (14) showed that in human liver preparations N,N-dimethylaniline is N-demethylated by cytochrome P-450 and N-oxidized by mixed function amine oxidase at approximately the same rate, thus demonstrating the high level of mixed function amine oxidase in the human liver. It is clear that mixed function amine oxidase is an enzyme of major importance in the human metabolism of drugs and other xenobiotics containing a basic nitrogen.

Surprisingly, few *in vitro* studies of this enzyme have been reported in the literature. This may be due in part to the difficulty in obtaining active enzyme preparations (15). In the studies described, we have investigated temperature induced conformational changes in the purified enzyme using intrinsic fluorescence and circular dichroism. Corresponding oxidative activity was determined using spectrophotometric techniques. At present, we are not able to correlate the observed structural changes with *in vivo* function; however, the moderate temperature at which the transition occurs would account for its unusual lability and might be of importance in the regulation of activity levels, *in vivo*.

MATERIALS AND METHODS

Purified mixed function amine oxidase from pig liver microsomes was a gift from Dr. Dan Ziegler, University of Texas, Austin, Texas. The enzyme was stored at −40° as a lyophilized powder from 0.1 M sodium phosphate buffer, pH 8.3, and diluted with water immediately before use. HEPES and methimazole were purchased from Sigma, L-tryptophan from Pierce Chemical, NADPH from Boehringer Mannheim and acrylamide and bis-acrylamide from Biorad. Other chemicals used were reagent grade. Deionized water was obtained from a system serviced and maintained by Continental Water. This water was free of interfering fluorescent impurities under the conditions employed.

[4] Rauckman and Rosen, unpublished results.

Fluorescence spectra were obtained on a Perkin Elmer MPF-3 spectrofluorimeter equipped with a R-446 photomultiplier tube. Temperature was controlled by means of a circulating water bath through the compartment cell holder. Temperature was measured by dipping a thermistor into a blank solution in the cell holder. Spectra are uncorrected. Polarization, P, was calculated from:

$$P = \frac{I_{vv} - \left(\frac{I_{hv}}{I_{hh}}\right) I_{vh}}{I_{vv} + \left(\frac{I_{hv}}{I_{hh}}\right) I_{vh}} \qquad (1)$$

where I is the observed fluorescence emission and subscripts v and h are vertical and horizontal orientation of the respective excitation and analyzer polarizers. The polarizers used were Polacoat, UV type 105.

Circular dichroism spectra were determined on a Jasco ORD-CD/5 modified by Sproul Scientific (Tucson, Arizona) for CD (SS 20 CD) modification. Spectra were recorded in a round quartz 1 or 10 mm path length cell with slit programmed to give a spectral band width of 1.0 nm.

Gel electrophoresis utilized an 8% polyacrylamide running gel and a 4% spacer gel, prepared according to Davis and Ornstein (16, 17). Gels were stained for 2 hr in Coomassie Brilliant Blue, then destained in a charcoal diffusion destainer. Removal of excess dye required several days.

The rate of methimazole or N,N-diethylaniline oxidation was determined by measuring the initial rate of NADPH oxidation at 340 nm using a Varian/Cary 118 spectrophotometer equipped with a thermostated cell holder. In a typical experiment, the reaction medium contained 1 mM methimazole or N,N-diethylaniline, 0.1 mM, NADPH, 0.1 ml purified mixed function amine oxidase (0.28 mg/ml) and sufficient buffer (0.1 M phosphate buffer at pH 8.3) to bring the final volume to 1.0 ml.

RESULTS

Fluorescence properties of mixed function amine oxidase. Mixed function amine oxidase (0.05–0.1 mg/ml, 0.1 M HEPES, pH 7.4 or 0.1 M sodium phosphate buffer, pH 8.3) has a fluorescence emission maximum near 325 nm when excited at 270–295 nm. The tyrosine contribution to the fluorescence is small, as shown by a difference method (18). The relative quantum yield, Q, of the enzyme is 0.02 (12°, 280 nm excitation) by comparison with $Q = 0.12$ for L-tryptophan in water (19). Fluorescence quantum yields of native proteins vary from 0 to about 0.3 (20), so the quantum yield for this enzyme is relatively low.

Effect of temperature on fluorescence properties of mixed function amine oxidase. As shown in Fig. 1, the fluorescence intensity of the enzyme at 325 nm decreased between 10 and 32° and again above 50°. A linear decrease is typical of many tryptophan-containing native proteins and is usually attributed to increased bulk solvent collisional quenching (21, 22). Between 32° and 50°, enzyme fluorescence intensity increased by 12%. If the enzyme was cooled to room temperature from 45°, 40°, or 36°, its fluorescence intensity increased significantly over that of the native enzyme (Fig. 1). Thus the changes in fluorescence intensity that the enzyme underwent at temperatures between 30–45° were irreversible. In addition, as temperatures of approximately 40° were attained, there was an irreversible red shift in emission wavelength maximum from 325 nm to 332 nm, also indicative of a change in fluorescing tryptophan environment.

When mixed function amine oxidase was heated to 36° or 40° for 15 min., and then cooled to room temperature, its far ultraviolet CD was irreversibly altered (Fig. 2). The change in CD was greater when the temperature was held at 40° than when 36° was maintained (changes of 17 and 11%, respectively); however, these results indicate that heating even to a moderate temperature causes an irreversible change in polypeptide backbone structure. Since this effect on the CD was concurrent with fluorescence changes, it appeared that the al-

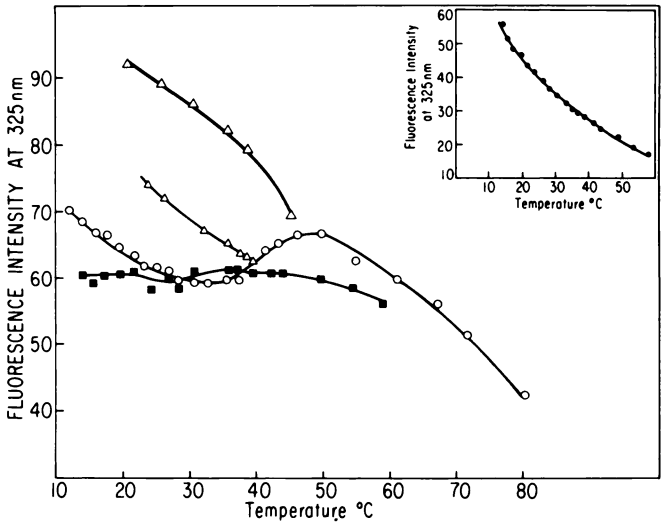

FIG. 1. *Fluorescence of mixed function amine oxidase, 0.1 mg/ml, in 0.1 M HEPES, pH 7.4, as a function of temperature.*

Heating curve (○), cooling curve (△) from 45° to 40° (similar, but not shown, is cooling curve from 36°); enzyme with 10-fold molar excess of NADPH (■). Inset shows effect of heating on a model compound, L-tryptophan in water. All emissions were monitored at 325 nm with excitation at 290 nm using a 290 nm cutoff filter. Not shown in this figure is the temperature-fluorescence curve for mixed function amine oxidase with methimazole: this curve can be superimposed on that of the enzyme. The enzyme curve is also unaffected by 1 mM glutathione, exclusion of oxygen, or change of pH to 8.3.

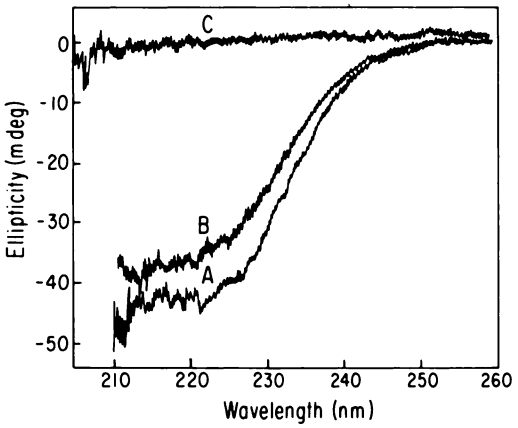

FIG. 2. *Effect of temperature on the far ultraviolet circular dichroism (CD) of mixed function amine oxidase, 0.4 mg/ml in 0.1 M sodium phosphate buffer, pH 8.3, 10 mm path length.*

A: at 29°; B: after heating to 40° for 15 min, then cooling to 29°; C: buffer alone.

teration in fluorescing tryptophan environment arose from the irreversible structural transition indicated by the CD.

Finally, the temperature fluorescence curve for the mixed function amine oxidase was not noticeably affected if oxygen was removed from the system before heating. This result indicated that the structural transition was not associated with oxygen binding. In addition, the temperature fluorescence curve for the enzyme was unaffected by the presence of 2 mM glutathione, indicating it is unlikely that irreversible sulfhydryl group oxidation was responsible for the observed transition. Also, the temperature-fluorescence curves were the same for the enzyme at pH 7.0 or 8.3, showing that the indicated structural change was not limited to the pH optimum (8.3) for enzyme activity.

The change in enzyme depolarization with temperature is shown in the Perrin plot in Fig. 3. Distinct breaks occurred near 34° and 38°, possibly due to structural changes. Since fluorescence lifetime, τ, was not determined, this experiment in itself did not prove that structural changes occurred at these temperatures.

Effect of temperature on other properties of mixed function amine oxidase. Ziegler has suggested that the native form of the enzyme is an octamer in equilibrium with small amounts of tetramer (15). Gel electrophoresis on 8% polyacrylamide at 25° and 36° showed identical band patterns for the enzyme after staining with Coomassie Brilliant Blue. This result suggested that the transition undergone by mixed function amine oxidase between 30–40° was not a change in multimer equilibria.

Effect of temperature on fluorescence properties of mixed function amine oxidase complexes with NADPH or methimazole. The mixed function amine oxidase temperature fluorescence curve remained virtually unchanged when 10-fold molar excess methimazole was added to the enzyme (Fig. 1). Enzyme fluorescence intensity was, however, affected when 10-fold molar excess NADPH was added. As shown in Fig. 1, fluorescence intensity of the mixed function amine oxidase-NADPH mixture at 325 nm showed little variation between 15° and 45°, in contrast to the intensity increase beginning at 30° when either the enzyme or the enzyme-methimazole mixture was heated.

Effect of temperature on methimazole oxidation by mixed function amine oxidase. When mixed function amine oxidase is incubated with NADPH and methimazole, a sulfur containing substrate, the course of thiol oxidation (7) may be followed by spectrophotometrically measuring the decrease in NADPH absorption at 340 nm. Figure 4 depicts an Arrhenius plot showing the rate of methimazole oxidation by mixed function amine oxidase as a function of temperature. This plot had a definitive break at about 33° where the calcu-

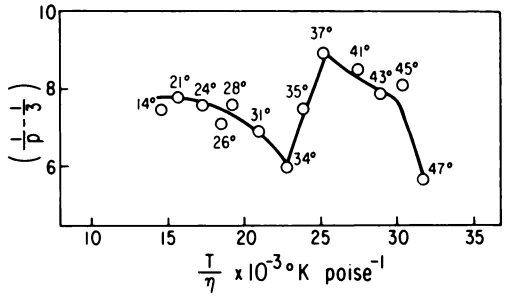

FIG. 3. *Fluorescence emission polarization of mixed function amine oxidase, 0.15 mg/ml, in 0.1 M sodium phosphate, pH 7.0 in 20% sucrose from 19–47° at 330 nm on 290 nm excitation.*

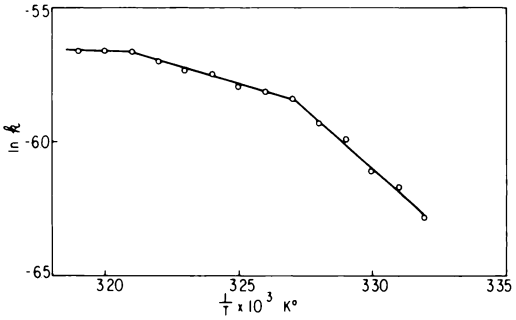

FIG. 4. *An Arrhenius plot of methimazole oxidation by mixed function amine oxidase, showing transitions at 33° and 38°.*

Experimental conditions are in MATERIALS AND METHODS.

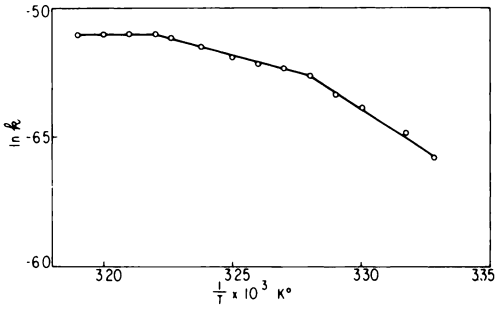

FIG. 5. *An Arrhenius plot of N,N-diethylaniline oxidation by mixed function amine oxidase showing transitions at 33° and 38°.*

Experimental conditions are in MATERIALS AND METHODS.

lated energy of activation, E_a, changed from 17.5 kcal/mol to 5.6 kcal/mol at higher temperatures. The corresponding entropy of activation, ΔS^*, changes from −12.8 cal/deg mol to −51.6 cal/deg mol.

To determine if the oxidative change was reversible, the enzyme was heated to 37° for 2 min, then rapidly cooled to 28°. The oxidative activity toward methimazole in the presence of NADPH was significantly reduced as compared to the unheated enzyme, showing that the change in oxidative activity was irreversible.

Effect of temperature on N,N-diethylaniline oxidation by mixed function amine oxidase. When mixed function amine oxidase is incubated with NADPH and N,N-diethylaniline, the course of N-oxidation may be followed spectrophotometrically by measuring the decrease in NADPH absorption at 340 nm. Figure 5 depicts an Arrhenius plot showing the rate of N,N-diethylaniline oxidation by mixed function amine oxidase as a function of temperature. There was a definitive break in the Arrhenius plot near 32° where the calculated energy of activation, E_a, changed from 12.4 kcal/mol to 5.67 kcal/mol at higher temperatures. The corresponding entropy of activation, ΔS^*, changed from −28.1 cal/deg mol to −50.2 cal/deg mol.

DISCUSSION

The effect of temperature on the intrinsic fluorescence properties of mixed function amine oxidase indicates that this enzyme is unusually heat labile *in vitro*. In the absence of conformational changes, tryptophan fluorescence emission normally decreases monotonically with increasing temperature as a result of temperature dependent bulk solvent collisional quenching (21-23). The insert in Fig. 1 shows this effect for a typical model compound, L-tryptophan. But the temperature fluorescence curve for mixed function amine oxidase indicates a transition between 30–40°, indicating that the fluorescing tryptophans undergo a change in microenvironment which affects fluorescence intensity and causes a small red shift in wavelength emission maximum. This appears to correlate with an irreversible loss of polypeptide backbone structure as shown by changes in the far ultraviolet CD for the enzyme. This structural change also correlates with an irreversible change in enzyme oxidative activity toward methimazole and N,N-diethylaniline near 33°. The data indicate that, *in vitro*, an irreversible structural change between 30–40° is the likely cause of the observed change in enzyme oxidative activity.

The structural transition that the enzyme undergoes at between 30–40° is unusual in several respects. There is a significant and irreversible loss of protein secondary structure. This backbone unfolding is typical for many native proteins, including enzymes, on heating; however, the process is frequently reversible and commonly occurs at significantly higher temperatures (21, 22,

24). The relatively low temperature of this structural transition for mixed function amine oxidase is possibly an artifact of the *in vitro* study. As an integral part of the hepatic microsomal system, the enzyme is membrane bound and may be stabilized by membrane lipids.

The detailed nature of the structural change between 30–40° is not known. Irreversible oxidative reactions involving enzyme sulfhydryls can be ruled out since we have shown that the temperature-fluorescence curve for the enzyme is not affected by the addition of glutathione. The change in degree of depolarization, $1/P$, with temperature, shows a sharp break near 34°. A significant structural change, e.g., denaturation or change in subunit association, could affect molecular volume and account for this change in depolarization, although other explanations are possible. Ziegler has suggested that the active form of the enzyme, *in vitro*, is an octamer in equilibrium with small amounts of tetramer (15). However, the results from disc gel electrophoresis at 25° and 36° indicate that no significant change in multimer equilibrium occurs. Unfortunately, this result is not conclusive since the effect of the gel on multimer equilibrium cannot be predicted.

Enzyme fluorescence emission begins to decrease near 45° and the enzyme visibly precipitates from dilute solutions. In the range 39–43°, the rate of enzymatic oxidation of methimazole and N,N-diethylaniline remains constant, suggesting progressive thermal denaturation, and possibly indicating a final structural alteration conducive to protein aggregation.

In the presence of NADPH, the temperature fluorescence curve of mixed function amine oxidase does not exhibit the transition shown in Fig. 1. However, since enzyme oxidative activity undergoes an abrupt change near 33° in the presence of substrates and NADPH, it is assumed that bound NADPH does not prevent the structural change, but does alter enzyme tryptophan fluorescence. The effect of NADPH upon the temperature-fluorescence curve of mixed function amine oxidase is possibly due to energy transfer between enzyme tryptophans and NADPH since there is some overlap between the tryptophan emission and NADPH absorption wavelengths (25).

Fluorescence and circular dichroism measurements have pointed to structural changes in mixed function amine oxidase between 30–40°. These data correlate well with definitive breaks in the Arrhenius plots for enzymatic methimazole and N,N-diethylaniline oxidation. The slope of the Arrhenius plot is related directly to the entropy of activation, ΔS^*, (26); thus, this transition results in an enzyme substrate complex which has a significantly lower ΔS^*. Since the function of an enzyme is to raise the entropy of activation for the reaction which it catalyzes, this transition results in a less effective oxidative conformation for the enzyme. This conformational change could play a major role in the regulation of substrate oxidation with the result that above 33° the enzyme conformation is less conducive to the oxidation reaction. Above 38°, temperature dependence on the rate of oxidation is entirely lost. This is likely due to further denaturation of the enzyme, a process that appears to be complete at approximately 45°, as indicated by visible aggregation. Thus a change in fluorescing tryptophan environment for mixed function amine oxidase between 30–40° has been ascribed to an irreversible structural change that correlates with a change in enzymatic oxidative activity.

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