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Kinetics and Mechanism of L-Tryptophan Oxidation by Chloramine-T in Basic Medium: A Spectrofluorometric Study

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Abstract One of the greatest challenges in oxidation research today is the determination of in vivo oxidative stresses. In this investigation, the spectrofluorometry is used to monitor the kinetics of chloramine-T (CAT) oxidation of L-tryptophan (Trp) in basic solutions. The Trp-CAT reaction progress has been monitored at λ_{max} = 485 nm (after using the excitation wavelength of 360 nm) over the temperature range, 293-313 K. The redox reaction follows a first-order dependence of the rate each on [CAT] and [Trp], and an inverse fractional-order on [OH-]. Variations of the ionic strength and the solvent dielectric constant have no effect on the rate. An addition of the reduction product of CAT, p-toluenesulfonamide, to the reaction mixture also has no influence on the rate. Based on the temperature effect, activation parameters are evaluated. A mechanism consistent with the observed kinetic and activation data has been proposed and the rate-law derived.

Keywords Oxidation, Reduction, L-Tryptophan, Chloramine-T, Spectrofluorometry, Mechanism, Rate Law

1. Introduction

One of the greatest challenges in oxidation research today is the determination of oxidative stresses in vivo. Under normal circumstances there is a well-managed balance between the formation and neutralization of free radicals, so that there is minimal modification of biomolecules. Free radicals are reactive oxygen species, which have the ability, either directly or indirectly to damage all biomolecules, including proteins, lipids, DNA and carbohydrates. They are formed in the course of normal metabolism through leakage of electrons from the electron transport chain and by the oxido-reductase enzymes. Under normal physiological conditions, cellular homeostasis is incessantly

Challenged by stressors arising from both internal and external sources[1-3]. To guard against these stressors, the cell has evolved its own defense mechanisms[4]. These involve mobilization of various cellular constituents and the functional integration of specific defense components[5], including membrane associated and cytosolic soluble free radical neutralizing and scavenging enzymes[6].

All biological oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, hypochlorous acid, and chloramines are products of PMS leukocytes and other phagocytic cells used to damage invading organisms in defense reactions of the host. In addition, they may be produced inadvertently, when stimulated in the course of inflammatory processes or other tissue damage, as PMN leukocytes react via an oxidative burst. Myeloperoxidase, which is activated and released from PMN leukocytes during stimulation, facilitates the reaction of H_2O_2 with Cl- to form H_2O and OCl-[7].

Hypochlorite can react with amines to form chloramines. Biological molecules such as aromatic amino acids, Trp and Tyr, can act as intrinsic fluorescent probes of protein conformation, dynamics and of intermolecular interactions.

N-haloaromaticsulfonamides containing a polar N-halogen bond act as mild oxidants. The diverse chemical behavior of N-haloamines has been attributed in general to their ability to act as halonium cations, hypohalites and N-anions, which behave both as electrophiles and bases[8]. As a result, these compounds react with a wide range of functional groups and effect an array of molecular transformations[8]. The prominent member of this class of compounds is sodium N-chloro-p-tolunesulfonamide or chloramine-T (CAT), which has diverse chemical properties and physiological importance. Chloramine-T has been used as a model agent for endogenously produced chloramines (N-Cl derivatives), which are formed as a result of the inflammatory process from myeloperoxidase secreted by stimulated monocytes and neutrophillic polymorphonuclear leukocytes[9]. The N-Cl derivatives are a class of long-lived oxidants produced by simulated phagocytes. Other phagocytes-generated oxidants have been shown to cause genetic damage in cultured mammalian cells. CAT has been

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used as a surrogate for the endogenously produced chloramines[9]. Chloramine-T oxidation studies involving many substrates have been reported[10-14]. However, the literature survey reveals that only a few studies are made from the kinetics and mechanistic view point on the oxidation of chosen aromatic amino acids by CAT. When the free radicals are the source of oxidative stress, it might be more useful to assay the products of tryptophan, tyrosine, methionine, and histidine as these amino acids are more susceptible to the attack by free radicals. In this investigation, fluorescence spectroscopic technique has been used to monitor the kinetics of L-Trp oxidation by CAT in phosphate buffered basic solutions.

2. Experimental

2.1. Materials

L-Tryptophan (99.9% purity) was obtained from Aldrich Chemical Co. and used as supplied. Phosphate buffer, 50 mM, pH 8, prepared from sodium phosphate monobasic and sodium phosphate dibasic salts, was used as the standard buffer system. Stock solutions of Trp were prepared in 50 mM phosphate buffer of pH 8. Concentrated aqueous solutions of CAT (Fluka Chemical Company) were prepared in amber colored bottles, iodometrically standardized, and stored as stock solutions. All other chemicals used were of analytical grade. Double distilled water was used in all preparations. A spectrofluorimeter (Systronics, S.R. No. 128) was used to measure the fluorescence.

2.2. Kinetic Procedure

Kinetic runs were performed under pseudo first order conditions of [CAT]0 >> [L-Trp]$_0$, Requisite amounts of solutions of L-Trp, NaOH, and the phosphate buffer were mixed in a stoppered Pyrex glass tube. The tube was thermostated in a water bath at 300 K. The reaction was initiated by adding a measured amount of pre-equilibrated, standard CAT solution. The reaction progress was monitored following changes in the fluorescence intensity at regular time intervals at the emission wavelength maximum of 485 nm after using the excitation wavelength of 360 nm using 50 mM phosphate buffer of pH 8 as a blank solution. The rate constant (k') of the reaction is calculated by plotting a graph of log of fluorescence reading vs. time.

2.3. Stiochiometry and Product Analysis

Reaction mixtures containing different compositions of L-Trp and CAT in the presence of 0.130 M OH^- were equilibrated at 300 K for 24 h. The iodometric determination of unreacted CAT in the reaction mixtures showed that one mole of L-Trp reacted with two moles of CAT. The reaction stoichiometry is represented by the equation,

$$\text{L-Tryptophan} + 2\text{TsNClNa} \xrightarrow{OH^-,\ H_2O} \text{Pyrrolo(2,3-b)indole} + 2\text{TsNH}_2 + 2\text{NaCl}$$

where chloramine-T is TsNClNa with Ts=p-$MeC_6H_4SO_2$.

The products in the reaction mixture were extracted several times with diethyl ether. The combined ether extract was evaporated and subjected to the column chromatography on silica gel (60-200 mesh) using gradient elusions. p-toluenesulfonamide (PTS or $TsNH_2$), in the reaction mixture, was identified by its mass spectrum obtained on a 70eV Shimadzu GCMS-QP5050 spectrometer which showed a parent molecular ion (M^+) peak at 171 amu. The formation of the oxidation product of L-Trp, pyrrolo (2,3-b)indole, was analyzed by its IR spectral data. The IR data showing the absence of carbonyl group band at 1700-1725 cm^{-1} indicated the product.

3. Results

The kinetics of oxidation of L-Trp by CAT was investigated under pseudo first-order conditions of [CAT]$_0$ >> [L-Trp]$_0$ at various concentrations of the reactants in basic solutions at 300 K.

Effect of CAT concentration on the rate:

When [CAT]$_0$ was varied keeping all other reaction conditions constant, linear plots of log (fluorescence) vs. time with nearly the same slope suggesting constant k' values were obtained (Figure. 1). This behavior indicated a first-order dependence of the rate on [CAT]. The values of k' are presented in Table 1.

Table 1. Effects of Varying Reactant Concentrations on the Reaction Rate

10^2 [CAT]$_o$ (M)	10^3[L-Trp]$_o$ (M)	10^3 k' (s^{-1})	Order*
0.52	3.48	1.71	
1.04	3.48	1.78	1.0 for CAT
1.56	3.48	1.83	
2.08	3.48	1.98	
2.60	3.48	1.93	
3.13	3.48	2.01	
2.08	0.348	0.17	
2.08	1.40	0.77	
2.08	1.74	0.992	1.0 for Trp (r = 0.9999)
2.08	3.48	1.98	
2.08	5.22	3.00	
2.08	10.4	5.70	

[OH^-] = 0.130 M; 50 mM phosphate Buffer; T = 300 K
Excitation λ_{max} = 360 nm; Emission λ_{max} = 485 nm
*From plots such as the one shown in Figure. 1.

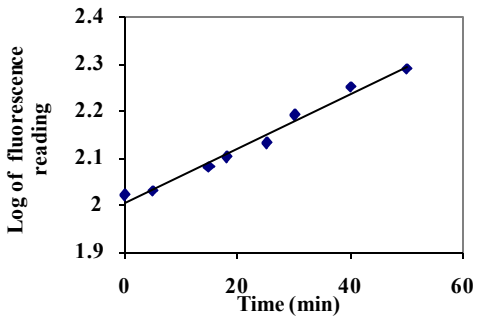
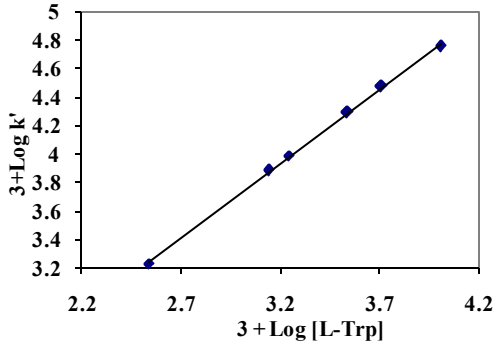


Figure 1. Representative first-order plot for the oxidation of L-Trp by CAT.[L-Trp]$_0$ = 3.48×10^{-3} M, [CAT]$_0$ = 2.08×10^{-2} M, [OH^-] = 0.130 M, T = 300K, Excitation λ_{max} = 360 nm, Emission λ_{max} = 485 nm

Effect of substrate (L-Trp) concentration on the rate:

At all other constant reaction conditions, the increase in the substrate Trp concentration ($3.48x10^{-4}$ to $1.04x10^{-2}$ M) increased the fluorescence of the CAT – L-Trp intermediate. A typical first-order plot of log (fluorescence reading) vs. time for a standard run, from the slope of which the pseudo first-order rate constant, k', value is determined, is presented in Figure. 1. Furthermore, a plot of log k' vs. log [L-Trp] was linear with a slope of unity indicating a first-order dependence of the rate on [L-Trp] (Table 1, Figure. 2).



$[CAT]_0 = 2.08\times10^{-2}$ M, $[OH^-] = 0.130$ M, T = 300K

Figure 2. Effect of varying concentrations of L-Trp on the reaction rate

Effect of hydroxide ion concentration on the rate:

The reaction was studied with varying [NaOH] with all other reaction conditions kept constant. The rate of the reaction decreased with an increase in [NaOH] (0.100 to 0.450 M) (Table 2). A plot of log k' vs. log [NaOH] was linear (Figure. 3) with a negative slope (-0.44) indicating an inverse fractional order on $[OH^-]$. A slight increase in the fluorescence intensity of the reaction with the variation of pH from 7.5 to 8.5 had a negligible effect on the reaction rate.

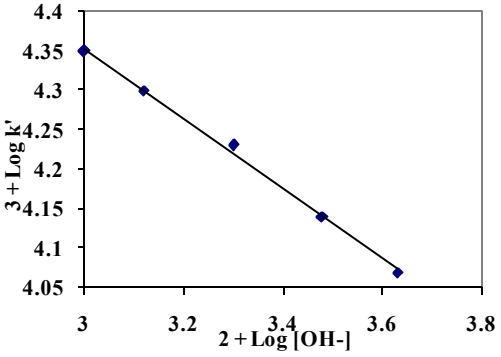


Figure 3. Effect of OH^- ion concentration on the oxidation of L-Trp.Reaction conditions are as in Table 2

Effect of chloride ion concentration on the rate:

The addition of chloride ions in the form of NaCl ($1.04x10^{-2}$ to $2.00\ x10^{-2}$ M) showed a slight increase in the reaction rate. This shows an approximately zero order dependence on $[Cl^-]$ (Table 2).

Effects of p-toulenesulfonamide (PTS) and MeOH on the rate:

The addition of PTS ($5.2\ x\ 10^{-3}$ M to $2.08\ x10^{-2}$ M; k' = $2.01\ x\ 10^{-3}$ to $2.03\ x\ 10^{-3}\ s^{-1}$) to the reaction mixture did not affect the reaction rate. The change in composition of the solvent with added MeOH (0-15% v/v) did not affect the k' value indicating that the dielectric constant (D) of the solvent medium did not have any effect on the rate.

Table 2. Effects of varying Hydroxide and Chloride Ion Concentrations on the Reaction Rate. $[CAT]_o = 2.08\ x\ 10^{-2}$ M; $[L\text{-}Trp]_o = 3.48\ x\ 10^{-3}$ M; 50 mM phosphate Buffer; T = 300 K; Excitation λ_{max} = 360 nm; Emission λ_{max} = 485 nm

$[OH^-]$ (M)	$10^2[Cl^-]$ (M)	10^3 k' (s^{-1})	Order*
0.100	0.00	2.25	
0.130	0.00	1.98	
0.200	0.00	1.68	-0.444
0.300	0.00	1.38	(r = 0.998)
0.425	0.00	1.18	
0.130	1.04	2.08	
0.130	1.26	2.12	~0
0.130	1.58	2.18	
0.130	2.08	2.22	

Effect of temperature on the rate:

Keeping all other conditions constant, the reaction temperature was varied in the range, 293-313 K, and the values of k' were determined at each temperature (Table 3). Arrhenius and Eyring plots (Figure. 4) were obtained from which activation parameters, namely, energy of activation E_a, enthalpy of activation $\Delta H^{\neq}$, and entropy of activation $\Delta S^{\neq}$ were calculated (Table 3).

Table 3. Temperature Dependence and Activation Parameters for the Oxidation of L-Trp by CAT in Basic Medium. $[CAT]_o = 2.08\ x\ 10^{-2}$ M; $[L\text{-}Trp]_o = 3.48\ x\ 10^{-3}$ M; $[OH^-] = 0.130$ M; 50 mM phosphate Buffer; Excitation λ_{max} = 360 nm; Emission λ_{max} = 485 nm

Temperature (K)	10^3k' (s^{-1})	
293	0.777	
298	1.12	
303	1.58	
308	2.21	
313	3.39	
E_a $\Delta H^{\neq}$ (kJ mol^{-1})	$\Delta S^{\neq}$ ($JK^{-1}mol^{-1}$)	$\Delta H^{\neq}$ (kJ mol^{-1})
57.1	-118.4	54.6

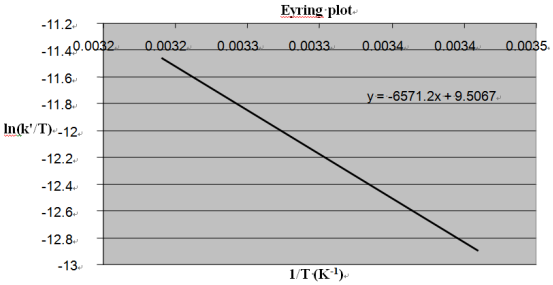


Figure 4. Eyring plot for the oxidation of L-Trp by CAT. Reaction conditions are as in Table 3

4. Discussion and Mechanism

In this study, CAT is used to mimic an oxidative stress produced in pathophysiological conditions. The oxidant enables monitoring of the oxidation of L-Trp by spectrofluorimetry. The kinetic data presented indicate that

L-Trp yields visible fluorescence when treated with CAT. The data show that the reaction leads to the visible fluorescence in two steps. The step 2 is influenced by phosphate or OH^- ions.

$$\text{Aromatic amino acid residues} \xrightarrow{\text{CAT}} \text{Intermediate X}$$

$$\text{Intermediate X} \xrightarrow{\text{base}} \text{Fluorescent product.}$$

The slight increase in pH indicates low fluorescence intensity which may be caused by the reaction with OH^- ions forming a non-fluoroscent compound. Furthermore, the slow increased reaction rate with increased concentration of chloride ions indicates the increased fluorescent intensity.

Pryde and Soper[15], Morries et al.[16], and Bishop and Jennings[17] have shown the existence of similar equilibria in acid and alkaline solutions of N- metallo- N- hallo-arylsulphonamides. Chloramine-T behaves as a strong electrolyte in aqueous solutions, forming different species as shown in equations (1) to (6) [8].

$$TsNClNa \rightleftharpoons TsNCl^- + Na^+ \quad (1)$$

$$TsNCl^- + H^+ \rightleftharpoons TsNHCl \quad (2)$$

$$TsNHCl + H_2O \rightleftharpoons TsNH_2 + HOCl \quad (3)$$

$$2TsNHCl \rightleftharpoons TsNH_2 + TsNCl_2 \quad (4)$$

$$HOCl + H^+ \rightleftharpoons H_2OCl^+ \quad (5)$$

$$HOCl \rightleftharpoons H^+ + OCl^- \quad (6)$$

In acid solutions of CAT, the probable oxidizing species are the free acid (TsNHCl), dichloramine-T ($TsNHCl_2$), $HOCl$ and H_2OCl^+. In alkaline solutions of CAT, $TsNCl_2$, and H_2OCl^+ do not exist. Therefore, the expected reactive species in basic medium are TsNHCl, HOCl, and $TsNCl^-$. The possible species $TsNCl^-$ and OCl^- could be transformed into more reactive oxidizing species TsNHCl and HOCl through reactions (eqs. 7-9).

$$TsNCl^- + H_2O \rightleftharpoons TsNHCl + OH^- \quad (7)$$

$$TsNCl^- + H_2O \rightleftharpoons TsNH_2 + OCl^- \quad (8)$$

$$OCl^- + H_2O \rightleftharpoons HOCl + OH^- \quad (9)$$

If OCl^- and HOCl were the primary oxidizing species as shown in equations (8) and (9), a first order retardation of the rate by the added $TsNH_2$ (p-toulenesulphonamide) would be expected. This is contrary to the experimental observations. A retarding influence of OH^- ions on the reaction rate has been observed in many reactions of CAT, which has been attributed to the formation of the conjugate acid TsNHCl from $TsNCl^-$ through reaction (7). Hence, a negative fractional order dependence of the rate on $[OH^-]$ observed here indicates the formation of TsNHCl as the most likely oxidizing species. Here the electron density around N atom in TsNHCl is decreased which weakens the N-Cl bond. The increased electrophillic character of TsNHCl facilitates the reaction with the substrate to form the intermediate complex X, which after wards gives the end products. In view of the above findings, it is likely that Scheme 1 is more suitable for explaining the oxidation of L-Trp by CAT in an alkaline medium.

$$TsNCl^- + H_2O \overset{K_1}{\rightleftharpoons} TsNHCl + OH^- \text{ fast} \quad \text{(i)}$$

$$TsNHCl + \text{L-Trp} \xrightarrow{k_2} X \text{ slow/rds} \quad \text{(ii)}$$

$$\text{(complex)}$$

$$X + H_2O \longrightarrow \cdots\cdots \longrightarrow \text{Products fast} \quad \text{(iii)}$$

Scheme 1. Oxidation of L-Trp by CAT

From the slow step in Scheme 1,

$$\text{Rate} = k_2\,[TsNHCl]\,[\text{L-Trp}] \quad (10)$$

If $[CAT]_t$ represents the total effective concentration of CAT in Scheme 1,

$$[CAT]t = [TsNCl^-] + [TsNHCl] \quad (11)$$

One gets,

$$[TsNCl^-] = \frac{[TsNHCl]\,[OH^-]}{K_1\,[H_2O]} \quad (12)$$

By substituting for $[TsNCl^-]$ in eq. (11) and solving for [TsNHCl], eq. (13)

$$[TsNHCl] = \frac{K_1\,[CAT]_t\,[H_2O]}{K_1\,[H_2O] + [OH^-]} \quad (13)$$

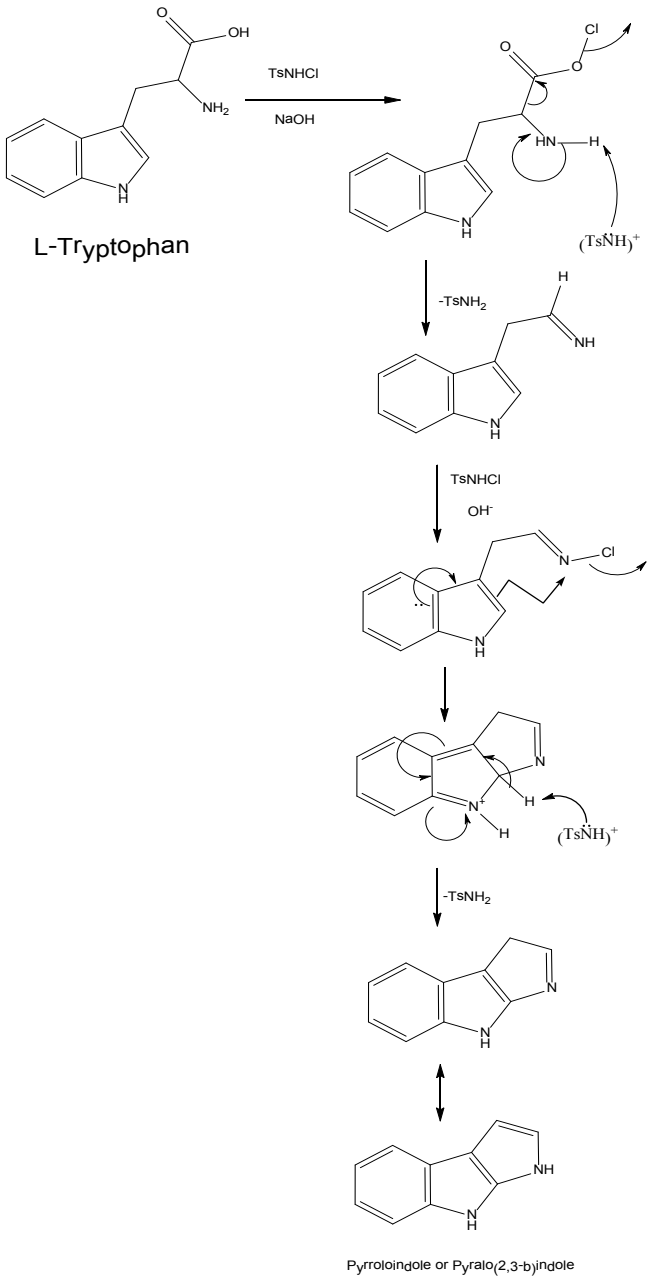
Substitution from eq. (13) into eq. 10) leads to the final rate law [eq. (14)],

$$\text{Rate} = \frac{-d[CAT]_t}{dt} = \frac{K_1 k_2\,[CAT]_t\,[\text{L-Trp}]\,[H_2O]}{[OH^-] + K_1\,[H_2O]} \quad (14)$$

This equation is in agreement with the experimental data including a first order dependence each on [CAT] and [L-Trp], and a negative fractional order on $[OH^-]$.

The electronic scheme showing the mechanistic steps, where the intermediate species including complex X shown in Scheme I, is presented in Scheme 2 below.

The results support the mechanism and most of the amino acids are susceptible to oxidation by the reactive oxygen species (ROS), while the final products may differ with the oxidation conditions[19]. Little is known about the physiological properties of Trp degradation compounds. Research using rat assays showed that the oxidation products formed by hydrogen peroxide treatment of free Trp were not utilized in protein synthesis[20] and lipids were oxidized while protein showed losses of bioavailable tyrptophan[21]. Oxidation products of Trp have been associated with the occurrence of cataractous lenses[22]. Several oxidation products of Tyrosine (obtained after exposing proteins or intact red blood cells to a flux of H_2O_2) are released by fragmentation or proteolysis after multiple complex reactions Viz., Cyclization, H-abstraction, oxidation and interaction with proximal amino acids[22-24]. The discussion mentioned above concludes that Tyr or Trp can be considered as a marker for biological systems exposed to oxidative stresses such as atherosclerosis, acute inflammation, systemic bacterial infections and lens cataracts[22].



Scheme 2. Electronic scheme for the Trp oxidation by chloramines-T in basic medium

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