[Chem. Pharm. Bull.]
[28(11)3437—3439(1980)]

Characterization of Aniline Hydroxylation by a Cytochrome P-450 Model and a Possible Hydroxylation Mechanism

HIROMU SAKURAI

Faculty of Pharmaceutical Sciences, University of Tokushima¹⁾

(Received May 19, 1980)

Aniline hydroxylation with a hemin-cysteine system was studied under various conditions as a model for cytochrome P-450 enzymes. The reaction was characterized by using various inhibitors, such as hydroxyl radical scavengers, a singlet oxygen trapping agent, superoxide dismutase and catalase. The system was compared with simple model systems consisting of cupric ion, ascorbic acid and hydrogen peroxide or the Udenfriend system. The hemin-cysteine model system was not inhibited appreciably by any of the inhibitors tested. On the basis of these findings and the fact that the thiolate-heme iron linkage was found to be present in the hemin-cysteine system, a possible mechanism for hydroxylation by the model system is proposed.

Keywords—cytochrome P-450 model; aniline hydroxylation; hemin-thiol complex; ascorbic acid; Udenfriend system; active oxygen; hydroxylation mechanism

Cytochrome P-450 is a heme-protein capable of activating dioxygen for its insertion into biomolecules and xenobiotics.²⁾ It is the only protein known definitely to have a cysteinate binding to heme iron. One of the fundamental problems about the mechanism of cytochrome P-450 enzymes is whether this cysteinate-heme iron linkage is retained during hydroxylations of substrates. Dawson *et al.* proposed a partial scheme for substrate hydroxylation involving a catalytically active oxygen intermediate, but they did not characterize the intermediate in the hydroxylation of the substrate with either cytochrome P-450 or a model system.³⁾

We previously demonstrated that a hemin-thiol compound system can be used as model of cytochrome P-450 in the hydroxylation of aniline,⁴⁾ *p*-toluidine⁵⁾ and other substrates⁶⁾ in aqueous acetone. Moreover, comparison with the results for synthetic model complexes showed that the system retains the thiolate-heme iron linkage in both the low-spin^{7,8)} and substrate-bound high-spin⁶⁾ forms. Here we present a possible mechanism of aniline hydroxylation by our model system in which the cysteine-hemin axial ligation is retained.

The hemin-cysteine system catalyzed the hydroxylation of aniline in the presence of excess cysteine,⁴⁾ and the yield of aminophenols depended on the pH of the solvent (Fig. 1) and the reaction time (Fig. 2). The present results show that a proton is necessary to activate molecular oxygen for the hydroxylation of aniline and that oxidation of cysteine to cystine controls the formation and decomposition of aminophenols. The various model systems

1) Location: *Scho-machi 1, Tokushima 770, Japan.*

2) G.S. Boyd, "Biological Hydroxylation Mechanisms," eds. by G.S. Boyd and M.S. Smellie, Academic Press, London and New York, 1972, pp. 1—9.

3) J.H. Dawson, R.H. Holm, J.R. Trudell, G. Barth, R.E. Linder, E. Bunnenberg, C. Djerassi, and S.C. Tang, *J. Am. Chem. Soc.*, **98**, 3707 (1976).

4) a) H. Sakurai and S. Ogawa, *Biochem. Pharmacol.*, **24**, 1257 (1975); b) *Idem*, *Chem. Pharm. Bull.*, **27**, 2171 (1979).

5) a) H. Sakurai and M. Kito, *Biochem. Pharmacol.*, **24**, 7647 (1975); b) *Idem*, *Chem. Pharm. Bull.*, **25**, 2330 (1977).

6) H. Sakurai, The 11th Symposium on Drug Metabolism and Action, Nagoya, 1979, pp. 33—36.

7) a) H. Sakurai, S. Shimomura, and K. Ishizu, *Chem. Pharm. Bull.*, **25**, 199 (1977); b) H. Sakurai, S. Shimomura, K. Fukuzawa, and K. Ishizu, *ibid.*, **26**, 1348 (1978); c) H. Sakurai, S. Shimomura, Y. Sugiura, and K. Ishizu, *ibid.*, **27**, 3022 (1979).

8) K. Asada, *Seikagaku*, **48**, 226 (1976).

Fig. 1. pH Dependency of Aniline Hydroxylation by the Hemin-Cysteine System

The concentrations were; hemin 1 mM, cysteine 100 mM, and aniline 100 mM in 10 ml of aqueous acetone solution (50% acetone). The reaction times were 2 hr at 40°. Each point represents the mean of four experiments $\pm$ standard deviation.

Fig. 2. Effect of Reaction Time on Aniline Hydroxylation by the Hemin-Cysteine System at pH 5.0

The conditions used were the same as in Fig. 1. Each datum is the mean of 2–3 experiments.

for hydroxylation of aniline are sensitive to inhibition by an inert gas atmosphere, hydroxyl radical scavengers⁸⁾ (such as potassium iodide, alcohol, dimethyl sulfoxide or formic acid), a singlet oxygen trapping agent⁹⁾ (1,3-diphenylisobenzofuran), superoxide dismutase⁸⁾ and catalase (Table I). In systems constaining ascorbic acid (AH_2), hydrogen peroxide (H_2O_2) and cupric ion (Cu^{2+}), and the Udenfriend system,¹⁰⁾ hydroxylation was inhibited by hydroxyl radical ($\cdot\text{OH}$) scavengers, a singlet oxygen ($^1\text{O}_2$) trapping agent or superoxide (O_2^-) dismutase.

TABLE I. Aniline Hydroxylation by Various Model Systems

Addition	Relative yield of aminophenols				
	[I] Hemin-Cysteine	[II] $\text{Cu}^{2+}\text{-AH}_2$	[III] $\text{Cu}^{2+}\text{-AH}_2\text{-H}_2\text{O}_2$	[IV] $\text{AH}_2\text{-H}_2\text{O}_2$	[V] (Udenfriend system) $\text{AH}_2\text{-H}_2\text{O}_2\text{-Fe}^{2+}\text{-EDTA}$
None	100	100	100	100	100
N_2	0	2.7	56	5.7	41
KI	83	0	1.5	4.5	54
DMSO	92	95	84	28	71
HCOONa	95	—	77	68	—
$\text{C}_2\text{H}_5\text{OH}$	100	—	82	66	—
DPIF	90	—	69	11	—
SOD	93	71	84	65	—
Catalase	94	71	—	—	—

Reaction conditions were as follows: reaction time, 2 hours; temperature, 31°; reaction pH, [I], 5.0; [II], [III], 4.5; [IV] 3.1; [V] 6.0. The yields of aminophenols from aniline were 1.05%, 5.58%, 8.07%, 2.45% and 5.33% in systems [I], [II], [III], [IV] and [V], respectively. The abbreviations used in this table are AH_2 , ascorbic acid; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethyl sulfoxide; DPIF, 1,3-diphenylisobenzofuran; and SOD, superoxide dismutase. The concentration were: hemin 1 mM; cysteine 100 mM and aniline 100 mM in the hemin-cysteine system. The concentrations were: AH_2 50 mM, Cu^{2+} or Fe^{2+} 5 mM, H_2O_2 25 mM, and aniline 80 mM. Bovine liver catalase (Sigma) 20000 units or SOD(Sigma) 1500 units was added to the reaction mixture (10 ml).

Khan *et al.*¹¹⁾ proposed that O_2^- was generated during the oxidation of ascorbic acid in the presence of cupric ions, while Hatchikian *et al.* and Van Hemmen *et al.*¹²⁾ demonstrated

9) T.C. Pederson and S.D. Aust, *Biochim. Biophys. Acta*, **385**, 232 (1975).

10) S. Udenfriend, C.T. Clark, J. Axelrod, and B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

11) M.M. Khan and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).

12) a) E.C. Hatchikian and Y.A. Henry, *Biochim.*, **59**, 153 (1977); b) J.J. Van Hemmen and W.J.A. Meuling, *Arch. Biochem. Biophys.*, **182**, 743 (1977).

the subsequent formation of $\cdot\text{OH}$ by a metal-ion-catalyzed Haber-Weiss reaction. As O_2^- is an obligatory intermediate in the activation of molecular oxygen in these model system, the active oxidant for hydroxylation is probably $\cdot\text{OH}$ or singlet oxygen. The hemin-cysteine system is not inhibited strongly by any of these inhibitors. Thus aniline hydroxylation in this system probably does not involve $\cdot\text{OH}$, O_2^- , $^1\text{O}_2$ or H_2O_2 , but may have a different reactive intermediate.

In view of the present findings and the facts that the hemin-cysteine system retains the thiolate-heme iron linkage and requires molecular oxygen, excess cysteine and an acidic pH for aniline hydroxylation, we propose that the hydroxylation sequence can be explained as follows. Thiolate-heme complex formed from the thiolate-hemin complex by reduction with excess cysteine reacts with molecular oxygen to form an intermediate in which oxygen is bound to the heme iron directly. The electron generated by the oxidation of cysteine to cystine is used to reduce the intermediate in our model system, then the oxygen bound to the thiolate-heme complex reacts with a proton to cleave the dioxygen bond and afford the reactive intermediate containing atomic oxygen. The heme iron in the intermediate is probably in equilibrium among various multivalent states. In this form, the thiolate group can push the electron through the heme iron to oxygen.³⁾ This clarifies the possible significance of retaining the thiolate-heme iron linkage in connection with the hydroxylation of aniline. The intermediate should itself be an active oxygen form. The proposed reaction cycle should continue in air until all the added cysteine has been oxidized.

Experimental

Hemin (Type I, bovine), superoxide dismutase (Type I, bovine) and catalase (bovine liver, 2x crystallized) were obtained from Sigma Chemical Co. 1,3-Diphenylisobenzofuran was from Aldrich Chemical Inc. Hydroxylation of aniline with model systems was carried out as reported previously.⁴⁾ The reaction products, *p*- and *o*-aminophenol (*p*- and *o*-AP) were determined by liquid chromatography.⁴⁾ Buffer solutions used were as follows: pH 3, 1 M HCl-1 M CH_3COONa ; pH 4-7, 1 M CH_3COOH -1 M CH_3COONa ; pH 7-8, 1 M tris(hydroxymethyl)aminomethane-0.5 M HCl; pH 8-9, 1 M NaCO_3 -1 M NaHCO_3 .

Acknowledgements This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Ministry of Welfare of Japan.

[Chem. Pharm. Bull.
28(11)3439-3441(1980)]

Synthesis of 5-(2-Amino-1-hydroxybutyl)-8-hydroxycarboxystyryl, One of the Major Metabolites of Procaterol

SHIRO YOSHIKAWA, EIYU YO, and KAZUYUKI NAKAGAWA

Laboratories of Medicinal Chemistry, Tokushima Factory,
Otsuka Pharmaceutical Co., Ltd.¹⁾

(Received May 30, 1980)

The two isomers of 5-(2-amino-1-hydroxybutyl)-8-hydroxycarboxystyryl (2), which is a major metabolite of procaterol, were synthesized.

Keywords—procaterol; metabolite; 5-(2-amino-1-hydroxybutyl)-8-hydroxycarboxystyryl; amination; reduction

1) Location: Kagasuno, Kawauchi-cho, Tokushima.