Gunsalus, R. P., & Wolfe, R. S. (1977) *Biochem. Biophys. Res. Commun. 76*, 790-795.
Gunsalus, R. P., & Wolfe, R. S. (1980) *J. Biol. Chem. 255*, 1891-1895.
Keltjens, J. T., Huberts, M. J., Laarhoven, W. H., & Vogels, G. D. (1983a) *Eur. J. Biochem. 130*, 537-544.
Keltjens, J. T., Daniels, L., Jannsen, H. G., Borm, P. J., & Vogels, G. D. (1983b) *Eur. J. Biochem. 130*, 545-552.
Leigh, J. A., & Wolfe, R. S. (1983) *J. Biol. Chem. 258*, 7536-7540.
Leigh, J. A., Rinehart, K. L., Jr., & Wolfe, R. S. (1984) *J. Am. Chem. Soc. 106*, 3636-3640.
Livingston, D. A., Pfaltz, A., Schreiber, J., Eschenmoser, A., Ankel-Fuchs, D., Moll, J., Jaenchen, R., & Thauer, R. K. (1984) *Helv. Chim. Acta 67*, 334-351.
Nagle, D. P., Jr., & Wolfe, R. S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2151-2155.
Pfaltz, A., Jaun, B., Fassler, A., Eschenmoser, A., Jaenchen, R., Gilles, H. H., Diekert, G., & Thauer, R. K. (1982) *Helv. Chim. Acta 65*, 828-865.
Romesser, J. A., & Balch, W. E. (1980) *Methods Enzymol. 67*, 545-552.
Romesser, J. A., & Wolfe, R. S. (1982a) *Zentralbl. Bakteriol., Abt. 1: Orig., [Reihe] C 3*, 271-276.
Romesser, J. A., & Wolfe, R. S. (1982b) *J. Bacteriol. 152*, 840-847.
Taylor, C. D., & Wolfe, R. S. (1974a) *J. Biol. Chem. 249*, 4879-4885.
Taylor, C. D., & Wolfe, R. S. (1974b) *J. Biol. Chem. 249*, 4886-4890.
van Beelen, P., Stassen, A. P. M., Bosch, J. W. G., Vogels, G. D., Guijt, W., & Haasnoot, C. A. G. (1984a) *Eur. J. Biochem. 138*, 563-571.
van Beelen, P., de Cock, R. M., Guijt, W., Haasnoot, C. A. G., & Vogels, G. D. (1984b) *FEMS Microbiol. Lett. 21*, 159-163.

Affinity-Dependent Cross-Linking to Neurotoxin Sites of the Acetylcholine Receptor Mediated by Catechol Oxidation†

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ABSTRACT: The choline homologue 3-[(trimethylammonio)methyl]catechol (TMC) has been synthesized, and the controllable features of its complex oxidation have been examined spectroscopically and correlated with its toxin binding inactivating reactions with the acetylcholine receptor (AcChR) from *Torpedo californica* electroplax. Affinity-dependent reactions of early intermediates in the oxidation of TMC are suggested to intercede covalently in this inactivation. At pH 7.4, where the oxidative polymerization of catechols proceeds spontaneously, pyrocatechol produced no effect on the toxin binding function of AcChR, whereas comparable concentrations of TMC led to inactivation of half of all available sites. Lower concentrations of TMC converted via oxidation with ceric salts to an in situ mixture of monohydroxylated catechols were shown to be effective in short-term incubations in inactivating approximately half of the toxin binding sites by covalent labeling of the receptor. Mixtures of dihydroxycatechol intermediates, hydroxy-*p*-quinones, and polymeric products led to nonspecific toxin binding site inactivation of AcChR in excess of half of all available sites. Collectively, the results suggest that both covalent labeling and oxygen reduction product inactivating mechanisms are operative in these model macromolecular site reactions and that catechol-containing affinity reagents may be useful in elucidating the molecular features of sites to which they are directed.

Impressive control mechanisms have evolved over the reactions associated with the biological oxidation of catechols. Their oxidation occurs spontaneously at physiological pH with half-lives for this autocatalytic, complex reaction ranging from minutes to hours depending on the structure of the catechol. It is generally believed that cells that contain high concentrations of catechols also possess the means of blocking or controlling their spontaneous oxidation [cf. studies on chromaffin granules by Njus et al. (1982, 1983)]. Both reduced molecular oxygen products and reactive, electrophilic quinones are formed in the course of spontaneous catechol oxidation, and there is evidence that both classes of products are toxic to cells (Jonsson, 1980).

Despite the toxicity of the reaction products, spontaneous or enzyme-catalyzed oxidation of catechols mediates a number of important biological processes. Cross-linking reactions between proteins and the reactive quinones generated are known or are suspected to underlie most of these phenomena, which represent either compartmentalized or extracellular polymerizations or limited cytotoxic reactions. The formation of the pigment polymers of melanin is perhaps the most well-known such example (Pawelek & Korner, 1982) while the reactions accompanying the hardening of barnacle and other arthropod secretions (Pryor, 1940; Lindler et al., 1973), the cell-mediated immune responses to the plant wax catechols derived from poison ivy, oak, or sumac (Symes & Dawson, 1954; Mayer, 1955; Liberato et al., 1981), and the defensive reactions of some plants toward an attacking virus (Mink,

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1965) afford examples that are less well-known.

The selectivity underlying quinone cross-linking reactions and the manner in which the biological oxidation of catechols is controlled toward that end are poorly understood. In the present effort we have studied the use of quinones derived from oxidation of a synthetic cholinomimetic tetraalkylammonium catechol in affinity-directed reactions with a specific protein site, the neurotoxin binding region of the nicotinic acetylcholine receptor (AcChR)[1] [for review, see Conti-Tronconti & Raftery (1982) and Maelicke (1984)]. As the extreme reactivity of catechols or the quinones produced in their oxidation would be attractive for biochemical studies, the goal has been to understand the features of catechol reactivity that may be controlled. The results reveal a unique reactivity pattern that may be experimentally manipulated and also illustrate the potential use of such reagents in the dissection of chemical features of interest within the macromolecular sites to which the catechol moiety is affinity directed.

Experimental Procedures

Synthesis of 3-[(Trimethylammonio)methyl]catechol Iodide. 2,3-Dimethoxybenzaldehyde (mp 49–52 °C) was obtained from Aldrich and was used without purification. All other chemicals were reagent grade. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected.

2,3-Dimethoxy-N,N-dimethylbenzylamine. 2,3-Dimethoxybenzaldehyde (1.0 g, 0.006 M) was added to a 40% solution of aqueous dimethylamine (30 mL, 0.453 M amine) and allowed to stand overnight at room temperature. Sodium borohydride (0.75 g, 0.22 M) was added to the clear solution with stirring for 30 min at room temperature. The solution was acidified and extracted several times with ether and then returned to about pH 12 by addition of sodium hydroxide, and the cloudy aqueous solution was exhaustively extracted. The former extract contained approximately 50 mg of 2,3-dimethoxybenzyl alcohol and was discarded. The latter extract was dried over anhydrous sodium sulfate and evaporated in vacuo to give a transparent, viscous oil, which could be used without purification in the next step. A small portion of the oily product was converted in near quantitative yield to the methiodide salt [3-[(trimethylammonio)methyl]veratrole (TMV), λ_{max} 282 nm, mp 164–165 °C] by treatment with excess methyl iodide in ether.

2,3-Dihydroxy-N,N-dimethylbenzylamine. The oil obtained from conversion of 2,3-dimethoxybenzaldehyde (1.0 g) to 2,3-dimethoxy-*N,N*-dimethylbenzylamine was taken up in 47% aqueous hydroiodic acid (15 mL, 0.054 M) and refluxed for 5 h. The excess hydroiodic acid was evaporated in vacuo and the black, oily residue taken up in an ice–water mixture and transferred to a separatory funnel containing ether. Sufficient sodium bicarbonate (10% solution) was added to raise the pH to near 10. Thereafter, the dark aqueous solution was extracted rapidly several additional times with ether (Note: it is important to rapidly extract the product, which is quite unstable at alkaline pH.). The combined ether extracts were washed once with 1% aqueous hydrochloric acid and saturated brine and then dried over anhydrous sodium sulfate. The evaporation of the ether in vacuo gave a light yellow oil that slowly turned dark brown on exposure to air. This unstable intermediate was routinely utilized immediately in the next step.

3-[(Trimethylammonio)methyl]catechol Iodide. The ether solution obtained in a typical preparation of 2,3-dihydroxy-*N,N*-dimethylbenzylamine from 1.0 g (0.006 M) of 2,3-dimethoxybenzaldehyde was reduced in vacuo to a volume of 20 mL, and an excess of methyl iodide (1.15 g, 0.0081 M) was added. The product crystallized on standing over the next 15 h and produced two crops (0.75 g) of small white crystals (40% of theory based on 2,3-dimethoxybenzaldehyde). An analytical sample obtained after recrystallization from ether–acetone had the following: mp 175–176 °C (uncorrected) [lit. mp (Epstein et al., 1964) 181 °C]; λ_{max}(pH 2) 287 nm (ϵ = 2100 M^{-1} nm), λ_{max}(pH 12) 302 nm. Anal. Calcd for $C_{10}H_{16}NO_2I$: C, 38.85; H, 5.21; N, 4.53; I, 41.05. Found: C, 38.57; H, 5.24; N, 4.29; I, 40.85.

[^{3}H]-3-[(Trimethylammonio)methyl]catechol Iodide. [^{3}H]Methyl iodide (25 mCi, 3.1 Ci/mM, 0.0081 mM) was vacuum transferred into a solution of 2,3-dihydroxy-*N,N*-dimethylbenzylamine (7.1 mg, 0.046 mM) in ether (1.0 mL). After the mixture was allowed to warm to room temperature and react for 1 h, a solution containing nonradioactive methyl iodide (0.03 mM) in ether (0.5 mL) was vacuum transferred into the cooled reaction mixture, which was subsequently warmed to room temperature and allowed to stand for 12 h. The product (8.9 mg, 0.75 Ci/mM) that crystallized was filtered and used in the experiments reported below without further purification. The specific activity (0.75 Ci/mM) was determined by counting aliquots of the prepared solutions of the reagent, the concentrations of which were determined from the weight used and checked by spectrophotometry.

Spectrophotometric, Acid–Base, and Oxidation Studies. All spectrophotometric data were obtained from a Beckman DBG or a Varian DMS-100 spectrophotometer. The pH of solutions was measured with a Radiometer 26 pH meter. Ceric ammonium nitrate was prepared in stock solutions of nitric acid and standardized by oxidation with iodide and titration of the iodine with thiosulfate. These solutions were stable for weeks; however, in most cases they were freshly prepared, standardized, and used the same day. In a number of instances, ceric ammonium nitrate was weighed out directly and simultaneously taken up into acidic solution with catechols. Absorption spectra were obtained under a variety of conditions and compared with those from oxidation of pyrocatechol and hydroxycatechol (Duckworth and Coleman, 1973; Dawson & Tarpley, 1960). Enzymatic oxidation of 3-[(trimethylammonio)methyl]catechol salts was observed with guinea pig tyrosinase (*o*-phenol:O_2 oxidoreductase) obtained from Worthington Biochemicals. The reaction was carried out in 0.1 M sodium phosphate, pH 7 (Jolley et al., 1972).

Acetylcholine Receptor: Purification and ^{125}I-Bungarotoxin Assay. Acetylcholine receptors from the electric organ of *Torpedo californica* (Pacific Biomarine, Venice, CA) were purified by affinity chromatography of the Triton X-100 solubilized electroplax by utilizing the principal toxin from the venom of the Thailand cobra, *Naja naja siamensis*, cross-linked to Sepharose 4B subsequent to activation of the latter by reaction with cyanogen bromide. The procedure was identical with that employed by Froehner & Rafto (1979) except that Sepharose 4B was used in place of Sepharose 6B. The neurotoxin was purified by the method of Karlsson et al. (1971) from the lyophilized venom, which was obtained from the Miami Serpentarium, Miami, FL. The quantity of neurotoxin loaded onto the affinity resin was quantitatively de-

[1] Abbreviations: TMC, 3-[(trimethylammonio)methyl]catechol; [^{3}H]TMC, [C$^\alpha$-^{3}H]-3-[(trimethylammonio)methyl]catechol; AcChR, acetylcholine receptor; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TMV, 3-[(trimethylammonio)methyl]veratrole; Btx, α-bungarotoxin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OBQ, *o*-benzoquinone; TMQ, 3-[(trimethylammonio)-methyl]-*o*-quinone.

termined from spectrophotometric differences in the toxin solution before and after mixing with the cyanogen bromide activated Sepharose (A_{280nm} = 1.06 for a 1.0 mg/mL solution; Karlsson et al., 1971). It was assumed that 10% of the cross-linked toxin effectively bound Triton X-100 solubilized receptor. Purification of the receptor was followed by SDS–PAGE on 7% acrylamide gels and by determination of increases in specific activity measured as ^{125}I-α-Btx binding sites per milligram of protein (Schmidt & Raftery, 1973). The separation of the receptors from acetylcholine esterase followed the procedure of Ellman (1961). Protein was determined by the method of Lowry (1951). ^{125}I-α-Btx was purchased from New England Nuclear. Some samples were passed through on a short column of DEAE-cellulose to minimize background counts (Lukasiewicz et al., 1978).

Rate and Extent of Inactivation of Btx Binding Sites by TMCI. The inactivation of the α-bungarotoxin binding sites of the acetylcholine receptor was determined with various concentrations of 3-[(trimethylammonio)methyl]catechol iodide (1–65 mM) prepared in receptor buffer (0.1% Triton-X-100, 10 mM sodium phosphate, pH 7.4) by using a gas train manifold to simultaneously incubate several solutions over a slight positive pressure of oxygen (95% oxygen, 5% carbon dioxide) at 5 °C for various intervals ranging from 2 to 48 h. The initial concentrations of AcChR were 0.05–0.10 mg/mL and had specific activities of 6.4–8.0 nM α-Btx sites/mg of protein. The AcChR was incubated alone or in the presence of pyrocatechol or TMC in the receptor buffer. The reduction of toxin binding sites per milligram of receptor was determined by the ^{125}I-α-bungarotoxin procedure noted above after extensive dialysis against receptor buffer to remove excess reagent. Control dialyses of catechols oxidized in the absence of AcChR were carried out to assure removal of toxin binding inhibiting polymers that may have been formed during the oxidation of catechols.

Concentrated solutions of 3-[(trimethylammonio)-methyl]-*o*-quinone and *o*-quinone were generated by oxidation of the corresponding catechol with 1 equiv of ceric ammonium nitrate in unbuffered nitric acid (pH 3), neutralized immediately and added to AcChR solutions containing 40–80 nM α-bungarotoxin sites. The final concentration of the catechol reagent ranged from 0.1 to 2.0 mM. After a given period of incubation, 2 mM DTT or 1% β-mercaptoethanol was added and the solution extensively dialyzed against the receptor buffer containing the corresponding thiol and analyzed for residual toxin sites. In some cases, no thiol was added.

Radioactive Labeling of the AcChR Using [^{3}H]TMC. The inactivation of toxin binding sites of the AcChR with [^{3}H]-TMC was determined by incubating a solution containing 45 nM toxin binding sites of AcChR with 22 mM [^{3}H]TMC. In controls, enough unlabeled toxin to saturate all bungarotoxin binding sites was added prior to [^{3}H]TMC. The [^{3}H]TMC had a specific activity of 0.75 Ci/mmol and was incubated with AcChR under standard conditions for 5 h and counted on DEAE-cellulose discs from which excess [^{3}H]TMC was washed. Incorporation of label into receptor was also followed after rapid chromatographic separation of excess [^{3}H]TMC and receptor on small DEAE-cellulose columns in experiments in which the [^{3}H]quinone was first generated from ceric ammonium nitrate in pH 3 nitric acid, neutralized, and added to receptor solutions in rapid succession as noted above.

Results

The chemical synthesis of 3-[(trimethylammonio)methyl]-catechol iodide (TMC, I) was carried out according to the scheme shown in Figure 1. This method was superior in yield,

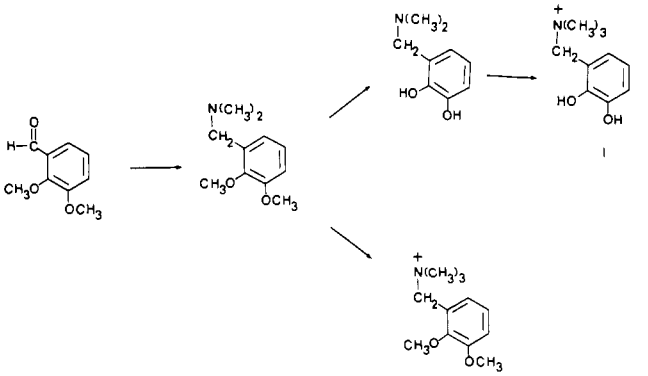

FIGURE 1: Chemical synthesis of 3-[(trimethylammonio)methyl]-catechol (TMC, I).

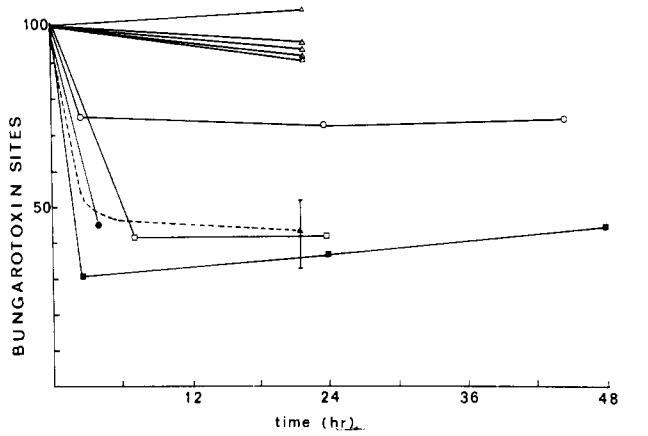

FIGURE 2: Kinetics of inactivation of ^{125}I-α-bungarotoxin binding sites of AcChR plotted as a percentage of the residual sites per milligram of protein against time of incubation with 3-[(trimethylammonio)-methyl]catechol [1 (○), 22 (●), 30 (▲), 43 (■), and 65 (□) mM]. Control incubations (△) for a single 21-h exposure in order of decreasing percentage of remaining sites: (a) TMC (30 mM) and hexamethonium (1 mM); (b) TMV (30 mM); (c) pyrocatechol (30 mM) and KI (30 mM); (d) buffer control; (e) receptor analysis carried after addition to a solution of autoxidized and dialyzed TMC (30 mM).

in ease of isolation, and in quality of the purified product when compared with a method of synthesis reported previously (Epstein et al., 1964). However, the rapid extraction of the [(*N*,*N*-dimethylamino)methyl]catechol intermediate from cold bicarbonate after hydroiodic acid cleavage of the methyl ether was critical to the purity of the final product. The method reported here also facilitated the preparation of a radiolabeled product through the use of [^{3}H]methyl iodide in the last step of the synthesis. A very brief account of the synthesis of unlabeled TMC and a preliminary account of its reaction with AcChR has been given elsewhere (Nickoloff et al., 1982).

To test the efficacy of I in affinity labeling a quaternary ammonium site, the reactions of TMC with AcChR have been examined. Purified receptor from *T. californica* was obtained in Triton X-100 by neurotoxin affinity chromatography. Various concentrations of TMC were incubated with AcChR for 3–48 h over an oxygen–carbon dioxide (95%/5%) atmosphere at 5 °C. After extensive dialysis, toxin binding site inactivation was measured as loss of ^{125}I-Btx binding sites/mg of AcChR as illustrated in Figure 2. The rate of toxin site inactivation was rapid and dependent on the concentration of TMC employed. Approximately 50% of all available sites were maximally inactivated at higher concentrations of TMC and this occurred before significant precipitating polymerization. At lower concentrations of TMC, the inactivation of toxin binding was slower and leveled off before half of all available

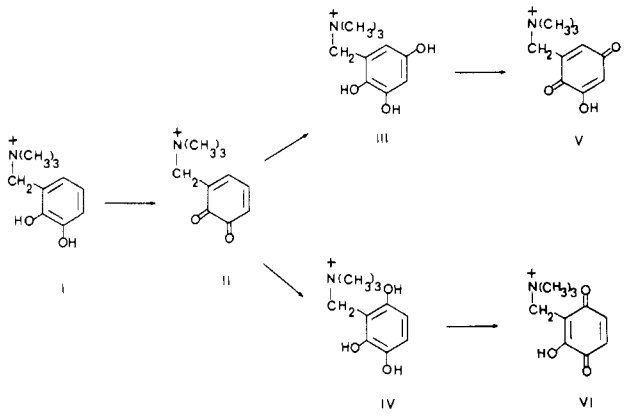

FIGURE 3: Major reaction sequence occurring early in the oxidation of 3-[(trimethylammonio)methyl]catechol (TMC, I). The products shown are 3-[(trimethylammonio)methyl]-*o*-benzoquinone (II), 5-hydroxy-3-[(trimethylammonio)methyl]catechol (III), 4-hydroxy-3-[(trimethylammonio)methyl]catechol (IV), 2-hydroxy-3-[(trimethylammonio)methyl]-*p*-benzoquinone (V), and 2-hydroxy-5-[(trimethylammonio)methyl]-*p*-benzoquinone (VI).

sites were inactivated. Control incubations included [(trimethylammonio)methyl]veratrole iodide and pyrocatechol with iodide. These showed little or no inactivation of Btx binding sites. To demonstrate that inhibition was not due to nondialyzable polymeric products, TMC was allowed to autooxidize in the absence of AcChR and extensively dialyzed. AcChR was then added to the dialyzate and shown to be unaffected in the analysis of the anticipated toxin binding sites per milligram of protein. No attempt was made to examine the effect of oxygen reduction intermediates directly as their formation was assumed to occur in the spontaneous oxidation of pyrocatechol. The binding constant for TMC with AcChR was previously shown to be $K_I = 2.8 \times 10^{-5}$ M, i.e., about 20-fold less well bound than hexamethonium (4.8×10^{-6} M) (Nickoloff et al., 1982). A 1 mM concentration of hexamethonium in the presence of 30 mM TMC was sufficient to completely block inactivation produced by 30 mM TMC alone. Collectively, these data suggested the inactivating species to be formed early in the spontaneous oxidation of TMC. To better understand the inactivating reaction as well as improve on its efficiency, we examined the early course of the oxidation of TMC spectroscopically.

The mechanism of the autocatalytic oxidation of catechols proceeds even in its first few steps in a relatively complicated fashion and by analogy with previous studies may be represented for TMC as shown in Figure 3. This early stage of the reaction may be controlled with the use of strong oxidizing agents in acidic solutions. A spectroscopic study of the intermediates depicted in Figure 3 is shown in Figure 4. The *o*-quinone II (λ_{max} 385 nm) may be easily observed in acidic solutions (cf. unsubstituted *o*-quinone, λ_{max} 390 nm, formed from pyrocatechol). Its decay in acid solution is shown in Figure 4a. Even at low pH there was a rapid reaction of the *o*-quinone via Michael addition with hydroxide or water to form mixtures of the trihydroxy compounds III and IV (Dawson & Tarplay, 1960). Elevated pH markedly increased the rate. The slow reaction to form III and IV in acid solution was followed by their rapid oxidative disproportionation with the unreacted *o*-quinone to form the *p*-quinones V and VI and TMC. Thus, in the presence of 1 equiv of ceric ion the spectrum of the initially formed *o*-quinones was observed to decay to that of the mixed *p*-quinones (V, VI). The kinetics of decay of the *o*-quinone and formation of the *p*-quinone species are shown in the inset in Figure 4a. Both rates are pseudo first order in the presence of a second equivalent of

Table I: Fraction of Remaining Btx Sites of the Btx of AcChR after Incubation with Neutralized Solutions of *o*-Quinones Generated in Acid Solution[a]

	1 mM OBQ	0.1 M TMQ	1 mM TMQ	2 mM TMQ
AcChR alone	0.99[b]	0.98	0.98	0.99
15-min incubation + dialysis	0.96	0.87	0.41	0.19
delayed addition for 15 min and then incubation for 15 min + dialysis			0.98	0.98

[a] OBQ and TMQ at the indicated concentrations were generated with 1 equiv of ceric ammonium nitrate neutralized and mixed immediately thereafter with AcChR. After incubation and dialysis, the remaining Btx sites were measured. [b] The fraction of remaining Btx sites divided by Btx sites before OBQ or TMQ addition.

ceric ion as well. The mechanism and kinetic treatment for the conversion of the *o*-quinone to *p*-quinone in acid solution in the presence and absence of a second equivalent of ceric ion are given in the Appendix. Subsequent to their maximal formation at acid pH from 2 equiv of ceric ion, the decay of the *p*-quinone spectra occurred over a much longer time interval than that of the *o*-quinone ($t_{1/2}$ = 11 h; cf. $t_{1/2}$ = 20 min for the decay of the *o*-quinone, Figure 4b). The insert shows the kinetics to be pseudo first order as well. Both *o*-quinones and mixed *p*-quinones may be trapped with thiols (Figure 4c) in reactions that were too rapid to be measured by conventional spectrophotometric techniques. Neither of these adducts nor any of the other intermediates suggested to be formed during the oxidation of TMC have been isolated and purified. Further details related to the mechanisms of the oxidative reactions of the TMC will be considered elsewhere. The above observations allowed us to devise conditions in which the early intermediates in the oxidation of TMC could be generated in higher concentrations than during the spontaneous oxidation. Further, the relative importance of the trihydroxy compounds (III and IV, Figure 3; cf. the quinone intermediates II, V, and VI, Figure 3) could be tested. The experiments reported below focus on the probable toxin site inactivating species.

When the first quinone species (II) was formed by oxidation of TMC in acid solution with 1 equiv of ceric ion and rapidly decomposed by neutralization just prior to incubation with the receptor, the rate of toxin site inactivation occurred rapidly with greater efficiency (Table I) but only if the neutralized solution was brought into contact with receptor within the first few minutes after neutralization. After incubation of the oxidized and neutralized mixture of TMC (1 mM) with AcChR for 15 min, mercaptoethanol or DTT was added, and the solution was extensively dialyzed against receptor buffer containing the corresponding thiol. Under these conditions, the trihydroxy intermediates (III and IV) are more rapidly formed (cf. reaction in acid solution) as the conversion of the *o*-quinone to III or IV is markedly elevated by increasing the pH whereas the oxidative disproportionation of III or IV with II is nearly pH independent (Dawson & Tarpley, 1960). The hydroxy intermediates under these conditions would be expected to have lifetimes of the order of minutes. Thus, the trihydroxy intermediates as well as the quinones appeared to be the inactivating species. The time course of inactivation was investigated (Figure 5). Half or less of the toxin sites were rapidly inactivated at concentrations of TMC in the 0.1–1 mM range as shown in Figure 5B. The extent of toxin site inactivation increased with time of exposure, an effect that is mimicked in AcChR solutions incubated with similarly

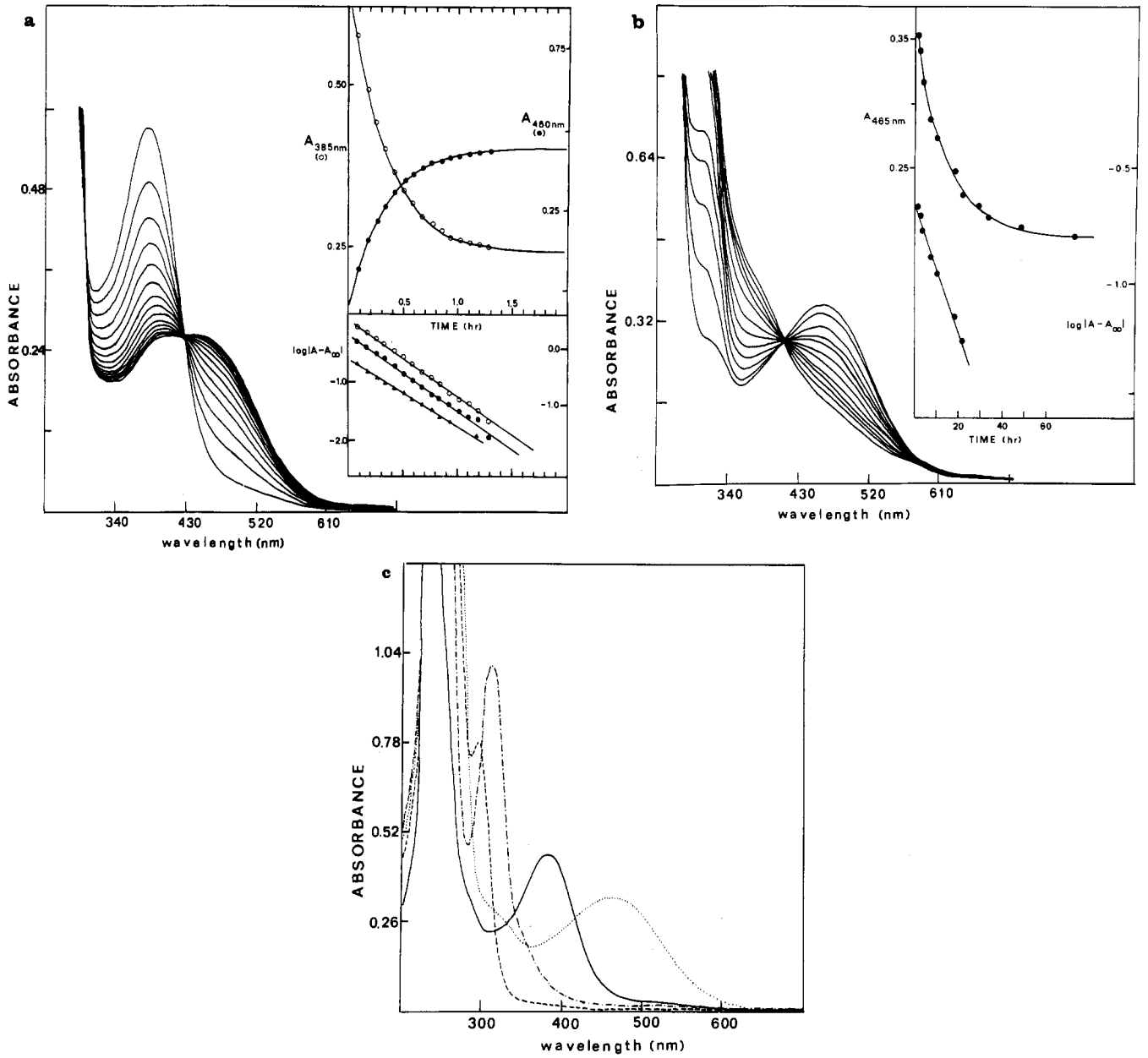

FIGURE 4: (a) Spectral plots with time for 3-[(trimethylammonio)methyl]-*o*-benzoquinone (II) formed from the oxidation of 3-[(trimethylammonio)methyl]catechol (TMC, 0.32 mM) with 1 equiv of ceric ammonium nitrate. The inset figure plots the absorbance decay of the initial maximum (λ_{385nm}) and rise at the new maximum (λ_{465nm}) against time. The first-order plots of log $|A - A_\infty|$ against time for both maxima are also shown in panel b together with a similar plot for the decaying spectrum at λ_{385nm} (▲) observed when 2 equiv of ceric ammonium nitrate was used to produce II. (b) Plots with time showing the decay of the spectrum of the *p*-benzoquinones V and VI formed subsequent to the decay of II produced initially from 2 equiv of ceric ammonium nitrate and 1 equiv of TMC. The inset plots the absorbance at 465 nm and log $|A - A_\infty|$ with time. (c) Spectra of the cysteine adducts with 3-[(trimethylammonio)methyl]-*o*-benzoquinone [(—) II; (– –) adduct] and the *p*-benzoquinone mixture [(···) V and VI; (–·–) adduct].

oxidized and neutralized solutions of pyrocatechol albeit at higher concentrations of the latter. Thus, a portion of the inactivation is nonspecific, appears to be alleviated by thiols, and is mediated by the quinones II, V, and VI as these are rapidly trapped by the added thiols. Since the inactivation of half-sites cannot be blocked by addition of thiols at the earliest time, we have concluded that the specific inactivating reactions mediated by TMC must in large measure be due to the trihydroxy intermediates III and IV. Addition of thiols to the receptor solutions just prior to mixture with the oxidized, neutralized TMC solutions did not affect the inactivation, but the absence of added thiol after incubation or in the dialysis buffer increased inactivation (cf. 1 mM TMC in Figure 5).

The covalent labeling of inactivated toxin sites was followed with [^{3}H]TMC. For example, incubation of the receptor with 22 mM [^{3}H]TMC for 3 h led to the labeling of 30–45% of the toxin binding sites that were inactivated. These percentages were calculated from counts incorporated above those observed when incubations of [^{3}H]TMC and AcChR were conducted in the presence of saturating concentrations of neurotoxin. These experiments were conducted by using DEAE-cellulose discs, to which the negatively charged receptor adheres at low ionic strength. Background counts, which were estimated by applying equivalent amounts of [^{3}H]TMC solutions allowed to oxidize in the absence of receptor or toxin, were somewhat lower than those observed in the presence of both toxin and receptor, indicating little nonspecific labeling of the AcChR in these experiments. In all cases most of the reactivity remained associated with [^{3}H]TMC or its oxidation products and was washed from the DEAE-cellulose disc. In

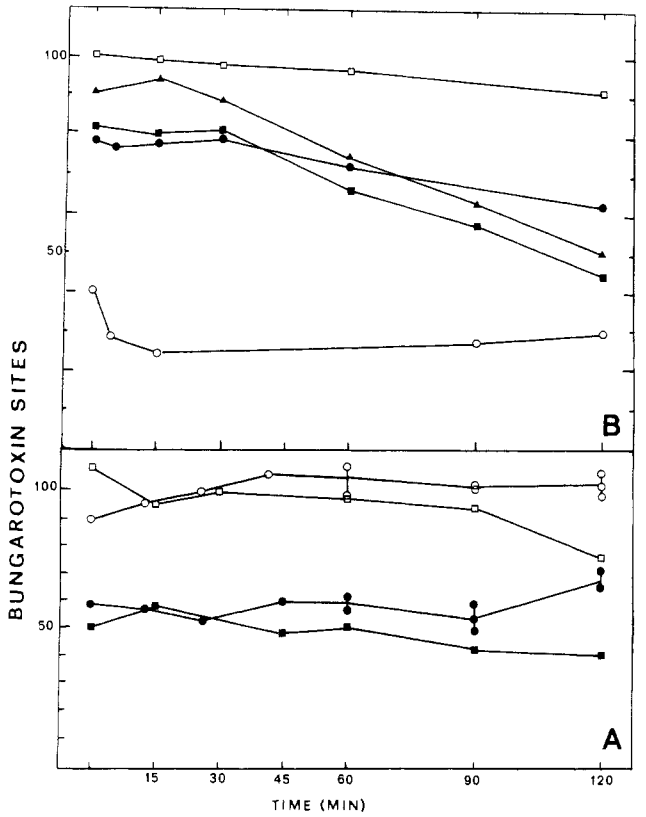

FIGURE 5: Inactivation of ^{125}I-bungarotoxin sites of AcChR plotted as a percentage of Btx sites per mg of protein remaining after incubation with the neutralized solution from TMC oxidized with 1 equiv of ceric ammonium nitrate. Panel A shows two such plots in which TMC (1 mM) oxidized and neutralized products were incubated with AcChR as a function of time prior to addition of 2 mM DTT (■) or 1% mercaptoethanol (●) and extensive dialysis with receptor buffer containing DTT or mercaptoethanol in the concentration indicated prior to analysis for residual neurotoxin sites. Two control plots in which mock neutralized solutions not containing TMC were added to AcChR solutions that were made 2 mM DTT (○) and 1% in mercaptoethanol (□) at the times indicated, dialyzed, and analyzed in a parallel way to the solution containing TMC. Panel B shows inactivation for TMC [0.1 (●), 0.5 (■), and 1.0 (○) mM] oxidized and neutralized products carried out as in panel A except that no thiols were added at the initiation of dialysis or to the dialysis buffer. Also shown are plots for pyrocatechol (1.0 mM) oxidized with 1 equiv of ceric ammonium nitrate and neutralized as with TMC (▲) and a receptor control constituted as in panel A but in the absence of added thiols (□).

separate experiments extensive dialysis to remove [^{3}H]TMC and oxidation products not bound to receptor resulted in the measurement of less radioactivity associated with the receptor. However, these results were highly variable.

By examining the labeling under conditions in which the inactivating reactions were more efficient, i.e., after incubation of the AcChR with oxidized and neutralized TMC (1 mM) for 15 min, the extent of labeling more nearly paralleled the extent of toxin binding inactivation. The mixture of reactants was rapidly chromatographed on DEAE-cellulose as shown in Figure 6. TMC oxidation products were eluted immediately or predominantly with the eluted receptor at 0.25 M NaCl in a step gradient. The radiolabel present in the eluted AcChR fractions represented labeling of ca. 40% of all toxin sites or about 80% of those apparently inactivated. These two types of labeling experiments taken together suggested that, while reaction of the reactive [^{3}H]TMC intermediate(s) with toxin binding sites was rapid and specific in the short term, the products of labeling were unstable, becoming detached from the AcChR in reactions that left the initially labeled sites permanently inactivated. Efforts to follow labeling of the

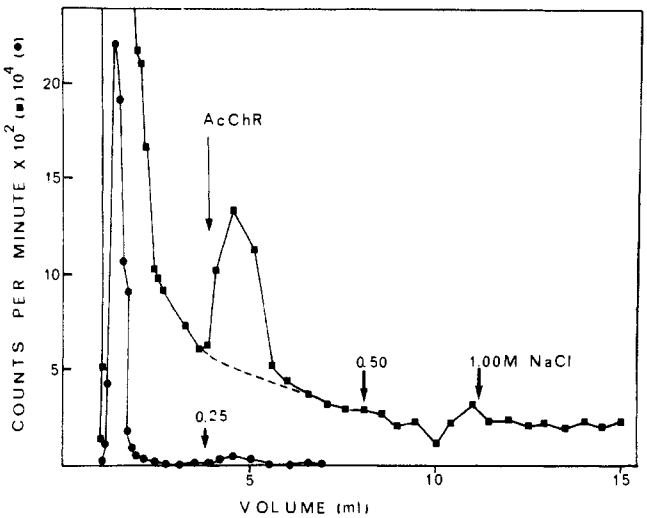

FIGURE 6: Separation of ^{3}H-labeled AcChR by discontinuous NaCl gradient DEAE-cellulose chromatography subsequent to 15-min incubation with 1 mM mixed 4- and 5-hydroxy-3-[(trimethylammonio)methyl]catechol iodide.

subunits by radioautographic analysis of dried SDS–PAGE gels have failed as there is accelerated loss of the label during sample manipulation.

DISCUSSION

Covalent labeling of proteins via quinones formed from catechols or *p*-dihydroxybenzenes is well-known (Sauer & Thoenen, 1971; Gupta & Vithayathil, 1980). Dopachrome and dopaquinones formed during melanogenesis react with the tripeptide glutathione and perhaps other peptides or proteins present in various cells in which melanin polymers are formed [cf. review by Pawelek & Korner (1982)]. The extent of the contribution of the peptide or protein to melanin polymer fractions differs depending on the site of melanogenesis. The insolubility of these polymers has made their quantitative analysis difficult at best, though coloration has been shown to qualitatively depend on the extent of protein or peptide involvement in this complex copolymerization (Prota, 1980). Similar difficulties arise in the analysis of the quinone-mediated protein copolymers formed during the hardening of barnacle and other arthropod secretions. There is little doubt, however, that the process is mediated by quinones (Lindley et al., 1973).

Covalent labeling via quinone is thought to be one mechanism underlying the neurotoxicity of 6-hydroxydopamine (Jonsson, 1981). The intracellular formation of this metabolite is evidently effectively blocked in catecholaminergic neurons by mechanisms that are yet to be described. There is also evidence to suggest that superoxide and hydroxy radical may be the actual cytotoxic agents (Heikkila & Cohen, 1971, 1973). The mechanisms involved are still open to question though superoxide, hydroxy radical, semiquinone radical, and singlet oxygen have all been implicated in the toxic response (Cohen & Heikkila, 1974; Heikkila & Cabbat, 1977; Felix & Sealy, 1981).

A number of enzyme or other protein inactivations have been linked to catechol oxidation. Covalent inactivation of tyrosinase by quinones is well-known (Duckworth & Coleman, 1970). Wick (1980) has suggested that inactivation of DNA polymerase occurs subsequent to incubation of tyrosinase-rich melanoma cells in culture with 6-hydroxydopamine and has proposed covalent labeling of an active site thiol to be the basis for this inactivation (Fitzgerald & Wick, 1983). Mink (1965) has suggested covalent inactivation of Tulare apple mosaic

virus proteins by quinones formed from the oxidation of chlorogenic acid, a catechol present in the plant cells attacked by the virus. Recently, Koshikara et al. (1984) have shown that caffeic acid metabolites, in which the catechol structure is maintained, are inhibitors of 5′-lipooxygenase, i.e., leukotriene synthesis inhibitors. It is possible that quinones may intervene here as well. Finally, the classic studies of Dawson and his collaborators (Symes & Dawson, 1954) on the delayed-type hypersensitivity reactions produced by the plant wax catechols of poison ivy, oak, and sumac have demonstrated the requirement of an oxidizable catechol with at least one site open for Michael addition by a protein nucleophile to the *o*-quinone oxidation product. However, the covalently modified proteins, which are presumed to mediate the complex delayed-type hypersensitivity cellular immune reaction, have never been isolated.

In addition to the present efforts, we are aware of only one series of studies in which an attempt has been made to use catechol oxidation products in affinity-dependent reactions. Borchardt & Bhatia (1982) have suggested affinity-dependent modification of rat liver catechol-*o*-methyl transferase on the basis of substrate protection and kinetic studies with the indole–quinone oxidation products from dihydroxyindolamines.

The results of the present study support a rapid affinity-dependent covalent inactivation of approximately half of the neurotoxin binding sites of the AcChR. While TMC is well designed in the sense that ring positions for the attack of external nucleophiles remain open in both the *o*-quinone and *p*-quinone oxidation intermediates (II, V, VI, Figure 2) and there is no possibility for the intramolecular nucleophilic inactivation, the reagent appears to be relatively inefficient. Several hundred- to a thousandfold molar excess of reagent is required to carry out the inactivation of half the neurotoxin sites. However, the rate and sensitivity of these sites toward inactivation is increased considerably if the first step in the reagent's oxidation is circumvented. Transient formation of the mixed trihydroxy intermediates, III and IV, allows inactivation of half of all toxin binding sites of the AcChR at lower reagent concentrations when compared with TMC. These observations suggest that the hydroxy intermediates must be formed before inactivation of toxin binding sites occurs, i.e., that the half-site inactivation resides in the trihydroxy intermediate or at some point subsequent to it in the spontaneous oxidation of TMC. The small effect of added thiols on the short-term inactivation of toxin binding sites by oxidized neutralized solutions of TMC suggests that neither the *o*-quinone (II) nor the *p*-quinones (IV and VI) are involved. However, these intermediates may intercede in the longer term nonspecific inactivation of the Btx binding site of AcChR. The enhanced selectivity of the ceric ion generated intermediates over those spontaneously formed may reside in the competitive inhibition of their binding by unreacted TMC during the slow spontaneous oxidation.

One mechanistic explanation for selective half-site inactivation and labeling mediated by the trihydroxy intermediates is suggested in Figure 7. The figure refers only to the reaction in which hydroxycatechol III participates. A completely analogous reaction scheme may be considered for hydroxycatechol IV.

The scheme is consistent with previous observations that have placed an easily reduced disulfide at half the toxin binding sites of the receptor (Damle & Karlin, 1978). The hydroxylated oxidation intermediates are proposed to mediate a site-dependent reduction of the easily reduced disulfide present at half the sites. The corresponding *p*-quinone so produced reacts

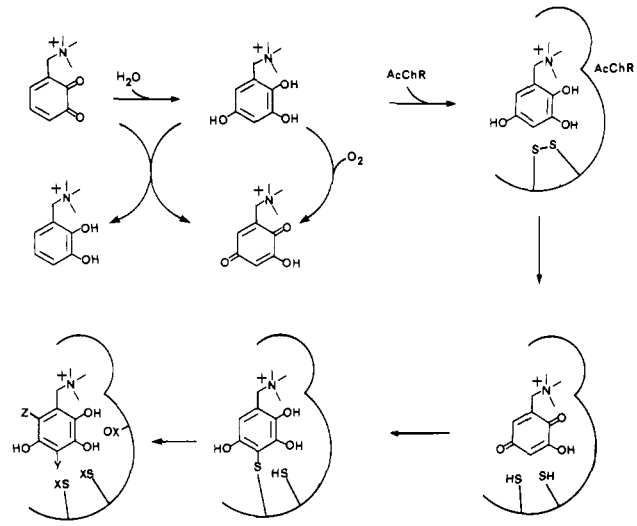

FIGURE 7: Possible scheme for the transient labeling and subsequent loss of label with oxidative inactivation of half of the neurotoxin sites of the AcChR.

with one of the pair of thiols to produce the covalently labeled protein. Subsequent oxidative inactivation of the site, which also features removal of the label, is then postulated to occur. The mechanistic details of this second phase process are left unspecified.

Site-specific oxidative inactivation is well-known. In a number of studies Ortiz de Mantellano and co-workers (Ortiz de Mantello & Kunze, 1981; Correia et al., 1981; Ortiz de Mantello et al., 1981; Ortiz de Mantello & Mathews, 1981; Augusto et al., 1982) have observed oxidative covalent inactivation of heme proteins. These covalent inactivations, which proceed through site-generated radicals and by more complex mechanisms, involve reaction of small molecule substrates with heme and less frequently with the side chains of neighboring amino acid residues. Godinger et al. (1983) have recently suggested a site-specific Fenton mechanism to explain ascorbate-enhanced cytotoxicity of metalloproteins. They propose a mechanism in which hydroxy radical is formed at the site of protein metal oxidation, accounting for the high efficiency of damage and the failure of hydroxy radical scavengers to effectively protect against its toxic effects. In the present context, the nature of the reactants and the discrete steps involved in the proposed oxidative removal of label from the protein remain to be determined.

In summary, these efforts have preliminarily characterized the chemistry involved in the covalent labeling and toxin binding site inactivating reactions of a new but not novel class of reagents. TMC and other reagents in this class may be applied to the site-directed inactivation of other enzymes or proteins that possess an affinity for the tetraalkylammonium function. The reagents and their chemistry described here also may form the basis for the design and synthesis of other heterobifunctional affinity reagents in which the catechol's oxidation may be employed in covalent modifications and inactivation of specific macromolecular sites.

APPENDIX

The pseudo-first-order kinetics observed in the disappearance of the *o*-quinone (II) as well as in the appearance of the *p*-quinone mixture (V and VI) is independent of the presence of 1 or 2 equiv of ceric ammonium nitrate. The similar kinetic behavior is consistent with different mechanisms for the conversion of II to V and VI as discussed for the two cases given below (cf. Figure 3 in the text).

Case A. The mechanism of decay of II and formation of V and VI in the absence of a second equivalent of Ce(IV) is

$$\text{II} + \text{H}_2\text{O} \xrightarrow[\text{slow}]{k_2} \text{III} + \text{IV}$$

$$\text{II} + \text{III} \xrightarrow[\text{fast}]{k_3} \text{I} + \text{V}$$

$$\text{II} + \text{IV} \xrightarrow[\text{fast}]{k_4} \text{I} + \text{VI}$$

Thus

$$-\text{d[II]}/\text{d}t = k_2[\text{H}_2\text{O}][\text{II}] = K_\psi[\text{II}]$$

where the pseudo-first-order rate constant

$$K_\psi = k_2[\text{H}_2\text{O}]$$

and

$$\text{d[V + VI]}/\text{d}t = k_3[\text{II}][\text{III}] + k_4[\text{II}][\text{IV}]$$

Assuming steady state for III and IV

$$\text{d[III + IV]}/\text{d}t = -\text{d[III + IV]}/\text{d}t$$

Then since

$$\text{d[III + IV]}/\text{d}t = K_\psi[\text{II}]$$

and

$$-\text{d[III + IV]}/\text{d}t = k_3[\text{II}][\text{III}] + k_4[\text{II}][\text{IV}]$$

$$K_\psi[\text{II}] = k_3[\text{II}][\text{III}] + k_4[\text{II}][\text{IV}]$$

and

$$K_\psi = k_3[\text{III}] + k_4[\text{IV}]$$

Consequently

$$\text{d[II]}/\text{d}t = \text{d[V + VI]}/\text{d}t = K_\psi[\text{II}]$$

and both the rates of decay of II and the rates of formation of the V and VI mixture are pseudo-first-order processes with equal absolute magnitude rates.

Case B. The mechanism of decay of II and the formation of V and VI in the presence of a second equivalent of Ce(IV) is

$$\text{II} + \text{H}_2\text{O} \xrightarrow[\text{slow}]{k_2} \text{III} + \text{IV}$$

$$\text{III} + \text{Ce(IV)} \xrightarrow[\text{fast}]{k_3} \text{V} + \text{Ce(IV)}$$

$$\text{IV} + \text{Ce(IV)} \xrightarrow[\text{fast}]{k_4} \text{VI} + \text{Ce(III)}$$

Thus

$$-\text{d[II]}/\text{d}t = k_2[\text{H}_2\text{O}][\text{II}] = K_\psi[\text{II}]$$

where K_ψ is equivalent to the pseudo-first-order rate constant cited for case A, and

$$\text{d[V + VI]}/\text{d}t = k_3'[\text{Ce(IV)}][\text{III}] + k_4'[\text{Ce(IV)}][\text{II}]$$

Assuming steady states for III and IV

$$\text{d[III + IV]}/\text{d}t = -\text{d[III + IV]}/\text{d}t$$

Then since

$$\text{d[III + IV]}/\text{d}t = K_\psi[\text{II}]$$

and

$$-\text{d[III + IV]}/\text{d}t = k_3'[\text{Ce(IV)}][\text{III}] + k_4'[\text{Ce(IV)}][\text{IV}]$$

$$K_\psi'[\text{II}] = k_3'[\text{Ce(IV)}][\text{III}] + k_4'[\text{Ce(IV)}][\text{IV}]$$

Consequently

$$-\text{d[II]}/\text{d}t = \text{d[V + VI]}/\text{d}t = K_\psi[\text{II}]$$

Also, for case B it should be noted that, since at the initial conditions where II is formed from 2 equiv of Ce(IV) and 1 equiv of I

$$[\text{II}]_0 = [\text{Ce(IV)}]_0$$

Thus, while in the initial stages of decay of II

$$K_\psi' = K_3'[\text{III}] + K_4'[\text{IV}]$$

However, even while K_ψ is approximately equal to K_ψ' experimentally, this relation does imply equality of K_3' and K_4' with the analogous K_3 and K_4 derived from case A. These respective rate constants would be equivalent only if the steady-state concentrations of III and IV were identical in the two cases but would differ in parallel to the extent that there were differences in the steady-state concentrations of III and IV.

Registry No. I, 1972-59-4; [^{3}H]-I, 94483-72-4; II, 94483-65-5; III, 94483-66-6; IV, 94483-67-7; V, 94483-68-8; VI, 94483-69-9; OBQ, 583-63-1; Btx, 11032-79-4; TMV, 94483-70-2; 2,3-dimethoxy-*N*,*N*-dimethylbenzylamine, 90704-69-1; 2,3-dimethoxybenzaldehyde, 86-51-1; dimethylamine, 124-40-3; 2,3-dihydroxy-*N*,*N*-dimethylbenzylamine, 94483-71-3.

References

Augusto, O., Beilan, H. S., & Ortiz de Mantellano, P. R. (1982) *J. Biol. Chem. 257*, 11288–11295.

Borchardt, R. T., & Bhatia, P. (1982) *J. Med. Chem. 25*, 263–271.

Cohen, G., & Heikkila, R. E. (1974) *J. Biol. Chem. 249*, 2447–2452.

Cohen, G., Heikkila, R. E., Allis, B., Cabbat, F., Dembiec, D., MacNamee, D., Mytilineou, C., & Wilson, B. (1976) *J. Pharmacol. Exp. Ther. 199*, 336–352.

Conti-Tronconi, B. M., & Raftery, M. A. (1982) *Annu. Rev. Biochem. 51*, 481–530.

Correia, M. A., Farrell, G. C., Olson, S., Wong, J. S., Schmid, R., Ortiz de Mantello, P. R., Beilan, H. S., Kunze, K. L., & Mico, B. A. (1981) *J. Biol. Chem. 256*, 5466–5470.

Damle, V. N., & Karlin, A. (1978) *Biochemistry 17*, 2039–2045.

Dawson, C. R., & Tarpley, W. B. (1960) *Ann. N.Y. Acad. Sci. 25*, 937–950.

Duckworth, H. W., & Coleman, J. E. (1970) *J. Biol. Chem. 245*, 1613–1625.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88–101.

Epstein, J., Michel, H. O., Rosenblatt, D. H., Plapinger, R. E., Stephain, R. A., & Cook, E. (1964) *J. Am. Chem. Soc. 86*, 4959–4963.

Felix, C. C., & Sealy, R. C. (1981) *J. Am. Chem. Soc. 103*, 2831–2836.

Fitzgerald, G. B., & Wick, M. M. (1983) *J. Invest. Dermatol. 80*, 119–123.

Froehner, S. C., & Rafto, S. (1979) *Biochemistry 18*, 301–307.

Godinger, D., Chevion, M., & Czapski, G. (1983) *Eur. J. Biochem. 137*, 119–124.

Graham, D. G., Tiffnay, S. M., Bell, W. R., Jr., & Gutknecht, W. F. (1978) *Mol. Pharmacol. 14*, 644–653.

Gupta, M. N., & Vitayathil, P. J. (1980) *Int. J. Pept. Protein Res. 15*, 236–242.

Heikkila, R. E., & Cohen, G. (1971) *Science (Washington, D.C.) 172*, 1257–1258.

Heikkila, R. E., & Cohen, G. (1972) *Mol. Pharmacol. 8*, 241–248.
Heikkila, R. E., & Cohen, G. (1973) *Science (Washington, D.C.) 181*, 456–457.
Heikkila, R. E., & Cabbat, F. S. (1977) *Res. Commun. Chem. Pathol. Pharmacol. 17*, 649–662.
Jonsson, G. (1980) *Annu. Rev. Neurosci. 3*, 169–187.
Karlsson, E., Arnberg, H., & Eaker, D. (1971) *Eur. J. Biochem. 21*, 1–16.
Koshihara, Y., Neichi, T., Murota, S.-I., Lao, A.-N., Fujimoto, Y., & Tatsuno, T. (1984) *Biochim. Biophys. Acta 792*, 92–98.
Liberato, D. J., Byers, V. S., Dennick, R. G., & Castagnoli, N., Jr. (1981) *J. Med. Chem. 24*, 28–33.
Lindler, E., Dooley, C. A., & Clavell, C. (1973) *Proceedings of the International Naval Conference on Marine Corrosion, 4th*, pp 358–391, Naval Research Center, San Diego, CA.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lukasiewicz, R. J., Hanley, M. R., & Bennett, E. L. (1978) *Biochemistry 17*, 2308–2313.
Maelicke, A. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 195–221.
Mayer, R. L. (1955) *Prog. Allergy 4*, 79.
Mink, G. I. (1965) *Virology 26*, 700–707.
Nickoloff, B. J., Grimes, M., Kelly, R., & Hudson, R. A. (1982) *Biochem. Biophys. Res. Commun. 107*, 1265–1272.
Njus, D., Knoth, J., & Zallakian, M. (1981) *Curr. Top. Bioenerg. 11*, 107–147.
Njus, D., Knoth, J., Cook, C., & Kelley, P. M. (1983) *J. Biol. Chem. 258*, 27–30.
Ortiz de Mantello, P. R., & Kunze, K. L. (1981) *Biochemistry 20*, 7266–7271.
Ortiz de Mantello, P. R., & Mathews, J. M. (1981) *Biochemistry 19*, 761–764.
Ortiz de Mantello, P. R., Mico, B. A., Beilan, H. S., & Kunze, K. L. (1981) *Biochemistry 19*, 151–166.
Pawelek, J. M., & Körner, A. M. (1980) *Am. Sci. 70*, 136–145.
Prota, G. (1980) *Invest. Dermatol. 75*, 122–217.
Pryor, M. G. M. (1940) *Proc. R. Soc. London, Ser. B 128*, 378–392.
Sauer, A., & Thoenen, H. (1971) *Mol. Pharmacol. 7*, 147–154.
Schmidt, J., & Raftery, M. A. (1974) *J. Neurochem. 23*, 617–623.
Symes, W. F., & Dawson, C. R. (1954) *J. Am. Chem. Soc. 76*, 2929–2963.
Wick, M. M., & Mui, A. (1981) *J. Natl. Cancer Inst. (U.S.) 66*, 351–354.

High-Resolution Proton Nuclear Magnetic Resonance Spectroscopy of Chloride Peroxidase: Identification of New Forms of the Enzyme†

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ABSTRACT: Chloride peroxidase from the mold *Caldariomyces fumago* in the native high-spin iron(III) and low-spin cyanoiron(III) states has been subjected to high-field proton nuclear magnetic resonance spectroscopic measurements. Signals shifted well outside the diamagnetic envelope by the paramagnetic iron(III) center are surprisingly insensitive to pH changes over the range from pH 3 to pH 7. The previously identified major form of chloride peroxidase (form A) and the minor form (B) show very similar chemical shift patterns. Of greatest significance, however, is the discovery that each of the separable forms of the enzyme exhibits splitting of porphyrin ring methyl resonances. The appearance of two sets of signals in both native and cyanide-complexed enzyme is best explained by the existence of two additional forms of the A and B isoenzymes. Structural differences for the newly identified forms of chloride peroxidase must be located in the vicinity of the heme prosthetic group.

High-resolution nuclear magnetic resonance (NMR) spectroscopy constitutes an especially powerful technique for elucidation of hemoprotein electronic and molecular structures. The utility of proton NMR spectroscopy relies on the very large signal dispersion for nuclei within the proximity of the paramagnetic heme center. Key signals for the prosthetic group and heme-pocket amino acid residues are thus distinguished from the hundreds of protein backbone resonances. Major classes of hemoproteins including hemoglobins, myoglobins, cytochromes, and peroxidases have been subjected to detailed proton NMR studies. Among the peroxidases, horseradish peroxidase (HRP) (Williams et al., 1975; Morishima & Ogawa, 1978; Morishima et al., 1977, 1978; La Mar & de Ropp, 1979, 1980, 1982; La Mar et al., 1980a–c, 1981, 1982a,b, 1983a–d), turnip peroxidase (Williams et al., 1975), and yeast cytochrome *c* peroxidase (Satterlee & Erman, 1980, 1981a,b; Satterlee et al., 1983a,b; La Mar et al., 1982a) have been examined by high-resolution NMR spectroscopy. Commercial availability of HRP has stimulated numerous studies of the native, iron(II), heme-reconstituted [iron(III)], and high-valent forms of this enzyme.

Proton NMR spectral measurments for chloride peroxidase (CPO) are reported here for the first time. This enzyme (EC 1.11.1.10) with a molecular weight of 42 000 is produced by the mold *Caldariomyces fumago* for biosynthesis of the

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