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Hydroxylation of Aniline by Hemin-Thiol Compound Solubilised by Non-Ionic Detergents: a Model System of Cytochrome P-450

THOMAS D. SMITH, RODNEY GAUNT and IVAN RUZIC

Chemistry Department, Monash University, Clayton, Vic. 3168, Australia

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Hemin chloride is solubilised in aqueous media by non-ionic detergents of the polyethylene oxide alkylamine type to give solutions which at neutral pH contain a dinuclear esr non-detectable form within the micelles of the detergent. Addition of β-mercaptoethanol effects the formation of iron(II) protoporphyrin IX and the resulting surfactant solution of this chelate brings about the hydroxylation of aniline and provides a model system for the function of cytochrome P-450.

Introduction

A wide variety of endogenous compounds and xenobiotics are metabolised by mixed function oxidases of the endoplasmic reticulum in a number of organs and tissues. Cytochrome P-450 is a generic name given to a group of heme proteins belonging to the class of mixed function oxidases which provides a major pathway for the elimination of fat-soluble compounds. Since its early discovery in the liver [1, 2] the enzyme has been found to be of ubiquitous occurrence in plant and animal physiology. Many aspects of the structure and function of cytochrome P-450 have been described [3]. The enzyme utilises one molecule of oxygen and two reducing equivalents to produce one molecule of water and the oxidised substrate through arene intermediates [4]. One atom of molecular oxygen is incorporated into the substrate and the other with the water as follows:

$$RH + 2e^- + O_2 \xrightarrow{2H^+} ROH + H_2O$$

The heme protein has a molecular weight of about 50,000 and is usually integrated into a membrane along with the electron transport system that provides the reducing equivalents. To reach the enzymatically active state cytochrome P-450 has a five-step reaction cycle the individual stages of which have been characterised by studies of the soluble cytochrome P-450 complex enzyme [5, 6]. Apart from studies of the enzyme, a number of investigations of the catalytic hydroxylation of organic compounds using heme compounds in the presence of sulphur containing ligands have been carried out. These studies have served as model systems to reproduce some spectroscopic or physicochemical properties of cytochrome P-450 and to draw some conclusions concerning the active site of the enzyme [7–11]. A major conclusion of studies of this type is that the spectroscopic characteristics of P-450 are similar to the iron(III) porphyrins having a thiolate axial ligand though the Fe–S bond of P-450 may be modified from its normal orientation in model thiolates possibly as a result of interaction with the protein structure. Other investigations have studied the circumstances whereby heme compounds may catalyse hydroxylation of substrate materials [12–20]. The evolution of model systems for the hydroxylation properties of cytochrome P-450 has been limited by the solubility properties of hemin, which is insoluble in water at pH 7 but dissolves when the pH exceeds 9.6. A number of studies have shown that hemin dissolves in aqueous solutions containing cationic, anionic and neutral detergent molecules over a wide range of pH [21–25], while the solubilization in water of iron(II) and iron(III) protoporphyrin(IX) by water soluble poly(aminophosphazenes) [26] and iron(II) protoporphyrin by poly(4-vinylpyridine), poly(vinylalcohol), poly(ethyleneoxide), polyvinylpyrrolidone and poly(styrene) sulphonate has been described [27–31].

In the present investigation the solubilisation of hemin in water by cationic, anionic, zwitterionic and non-ionic detergents was studied. Of the many samples of each type of detergent that were tried the non-ionic detergents, which possess an alkylamine polyethyleneoxide structure, were found to be the most satisfactory, while the cationic, anionic and zwitterionic materials were less satisfactory, requiring the presence of a larger amount of detergent to effect the solubilisation of relatively small amounts of hemin in water. Even so of the more than 40 samples of detergent materials tried, only six were able to solubilise hemin in water in sufficient quantity for subsequent work. It is interesting to note that non-ionic detergents of the polyethylenoxide type combine good solubilising properties with minimal damage to the heme protein cytochrome P-450

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regardless of its source [32]. Accordingly, aqueous solutions of hemin in the nonionic detergents Teric 16M15 and Teric 18M15 were used as model circumstances for the hydroxylation properties of cytochrome P-450.

Experimental

Materials and Apparatus

The non-ionic detergents, Teric 16M10 and Teric 16M15 were obtained from ICI.ANZ Pty. Ltd. The designation 16M15 refers to 16 carbon atoms in the alkylamine chain while 15 refers to the number of ethyleneoxide units. β-mercaptoethanol was purchased from Aldrich Chemicals while crystalline iron-(III) protoporphyrin IX chloride (hemin chloride bovine type I) was obtained from Sigma, St. Louis. All organic solvents were distilled freshly before use. The aniline was freshly distilled at diminished pressure. The u.v.–visible spectra and spectrophotometric measurements were made with a Varian Techtron Model 635 spectrophotometer. All pH measurements were taken with a Radiometer model 22 pH meter fitted with a glass electrode and a saturated calomel electrode system and calibrated with phthalate and borate buffer solution. A thermostatically controlled water bath, set at 40 °C was used for the temperature control of the aniline hydroxylation reactions. All e.s.r. measurements were taken with a Varian E-12 spectrometer, with samples at the temperature of liquid nitrogen.

Hydroxylation of aniline

The procedure for the hydroxylation of aniline by the hemin-detergent β-mercaptoethanol system was similar to that used by Sakurai [12–19]. In a typical experiment the reaction mixture contained hemin chloride (1.0×10^{-3} *M*) detergent (4% w/v), β-mercaptoethanol (1.0×10^{-1} *M*), aniline (1.0×10^{-1} *M*) and in some cases imidazole (1.0×10^{-3} *M*). The reaction mixture (10 ml total volume) was exposed to air with vigorous shaking at 40 °C for 1 hour. The pH of the reaction mixture was 7.2 and the reaction stopped, after 1 hour shaking, by addition of 2 *M* hydrochloric acid (0.5 ml). The amino-phenols formed in the reaction were extracted from the reaction mixture by a modification of the method outlined previously [33–36]. The reaction mixture was saturated with sodium chloride and the pH adjusted to 7.0 and extracted with diethyl ether (25 ml). The separated ether layer was extracted with 0.1 *M* hydrochloric acid (5 ml). The aqueous phase, now containing the amino-phenols, was washed with an equal volume of chloroform to remove any remaining aniline. The aqueous solution of amino-phenols were further purified by the use of column chromatography [35, 36]. The aqueous samples (5 ml) were applied to column of Sephadex G10. 0.1 *M* phosphate buffer, pH 3.0 was used as the mobile phase and 3 ml fractions collected automatically and used for U.V. spectroscopic analyses. The column was calibrated using standard solutions of ortho and para-amino-phenols. A known volume of the solution of the isolated amino-phenols was applied to a silica-gel thin layer chromatography plate and separated through the use of a mobile phase consisting of chloroform, iso-propyl alcohol and ammonium hydroxide in the ratio 16:3:1. The amino-phenol spots were removed by treatment with ethanol and the amount of each isomer determined by U.V. spectrophotometry by comparison with standard amounts of each isomer.

Results

An aqueous solution of iron(III)protoporphyrin-(IX) chloride(hemin) was prepared by dissolution of the hemin in an aqueous solution containing 4% of the non-ionic detergent Teric 18M15. The resulting green solution is characterised by its electronic U.V.–visible absorption spectrum which possesses a Soret absorption at 399 nm and the very much weaker α and β bands at 600 nm and 570 nm respectively. For comparison the green solution of hemin in 0.1 *M* sodium hydroxide has a Soret absorption at 390 nm with α and β bands at 599 and 573 nm respectively. When an excess of imidazole or pyridine is added to the green solution of hemin in aqueous solution containing the surfactant the colour changes to red (Soret 415 nm). Dilution of this solution results in a diminution of the red colour and a return of green colouration, immediately pointing to an axial interaction of base with the iron(III) chelate and ruling out the remote chance of an auto reduction process [37]. When the pH of aqueous-surfactant solution of hemin was varied from pH 8.4 (λ_{Soret} = 399 nm) to pH 1.5 the Soret band moves to 386 nm, the superposition of the appropriate visible absorption spectra showing isosbestic points at a number of wavelengths. Again the lowering of the pH of the aqueous-surfactant solution of hemin containing imidazole (pH 8.5 λ_{Soret} = 415 nm) caused a progressive shift of the Soret band to 399 nm. The esr spectra was recorded at 77 °K for samples of all the solutions. A signal was observed at g = 6 when the pH of the aqueous-surfactant solution of hemin was low, (about pH 1.5). This signal became progressively weaker as the pH was raised being barely discernable at pH 5.0. The addition of imidazole to the aqueous-surfactant solution at pH 8.5 though causing a colour change did not result in the occurrence of resonance in the esr spectrum.

The variation of pH conditions combined with various possibilities of axial interactions conspire to

lead to a variety of iron(III) protoporphyrin species in solution, which includes monomeric and dinuclear species [38]. The observation of a resonance at g = 6 in the esr spectra due to aqueous-surfactant solutions of the iron(III) porphyrin at low pH is evidence for the existence of the iron(III) chelate in a monomeric form presumably with axial ligation by water. The disappearance of the esr signal with rising pH along with concomitant changes in the U.V.–visible spectra occurs as a result of the magnetic coupling of the unpaired electrons of the coordinated iron(III) and occurs as a result of the formation of polymeric or dimeric species or dinuclear species involving μ-oxo bridging [41, 42].

Hemin chloride solubilised by the non-ionic detergent, is readily reduced by β-mercaptoethanol. The separate addition of sodium borohydride, ascorbic acid or sodium dithionite also effects a reduction of hemin; however under these solution conditions it is required that these reagents be present in much greater amounts, typically ten-fold excess of the amount of hemin present.

The aqueous non-ionic detergent solution of iron-(II) protoporphyrin IX, produced by reduction of the hemin by β-mercaptoethanol brings about a catalytic hydroxylation of aniline when the reaction mixture is exposed to air. Typical reaction conditions for the hydroxylation of aniline consist of an aqueous solution (10 ml) containing hemin chloride (1.0×10^{-3} *M*), non-ionic detergent (4% w/v) β-mercaptoethanol (1.0×10^{-1} *M*), aniline (1.0×10^{-3} *M*) and in some cases imidazole (1.0×10^{-3} *M*). The reaction mixtures, where pH was about 7.2, were maintained at 40 °C, and with vigorous shaking so as to allow sufficient contact with air. After about an hour the reaction was stopped by addition of dilute hydrochloric acid (0.5 ml, 2 *M*). The products of the hydroxylation of aniline, the isomers of amino-phenol, namely the ortho and para-amino-phenol, were separated from the reaction mixture and from one another and assayed separately to determine the ratio in which they occur in the product mixture.

In keeping with the findings of other studies there is no m-amino-phenol formed during the hydroxylation of aniline in the circumstances described here, where the hydroxylation reaction requires the presence of solubilised hemin, β-mercaptoethanol, and molecular oxygen, circumstances which correlate with the *in vivo* activity of cytochrome P-450. The major results obtained are summarised by Table I, which show that the amount of amino-phenol at pH 7.4, close to physiological conditions, is similar to that reported for the other model systems.

Discussion

The solution circumstances of hemin chloride in the aqueous non-ionic detergent solutions favour the formation of a dinuclear species which is stable in the presence of imidazole but which gives rise to monomeric species at low pH. The introduction of β-mercaptoethanol reduces the hemin and provides conditions for the hydroxylation of aniline when the reaction mixture is exposed to air at 40 °C. The hydroxylating properties of the system correspond with those achieved by cytochrome P-450 under physiological conditions, where the micelles of the non-ionic detergent possesses some of the properties of the protein. It is therefore interesting to note that the structural change to the micelle brought about by change in the surfactant molecular structure, namely a change in the number of ethylene oxide units, results in a measurable difference in the para to ortho ratio of the amino-phenols.

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TABLE I. Hydroxylation of Aniline by the Hemin to Non-ionic Detergent Model Systems. The pH of all solutions adjusted to 7.4.[a]

System	*p*-aminophenol (μg)	*o*-aminophenol (μg)	Total (μg)	*p*/*o* ratio
1. Hemin chloride + β-mercaptoethanol in aqueous Teric 16M10.	56 ± 5	36 ± 3	92	1.56
2. Hemin chloride + β-mercaptoethanol in aqueous Teric 16M15.	57 ± 5	46 ± 4	103	1.25
3. Same as mixture 1 + imidazole.	36 ± 4	25 ± 3	60	1.44
4. Same as mixture 2 + imidazole	35 ± 4	25 ± 3	61	1.40

[a]The limits of uncertainties were determined from measurements on 5 different solutions for each reaction mixture.

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