Inorganica Chimica Acta, 37 (1979) 141–144

Bioinorganic Chemistry Paper

Aqueous Ferric Chloride as a Model for Peroxidase in the Catalysed Autoxidation of Indole-3-acetic Acid

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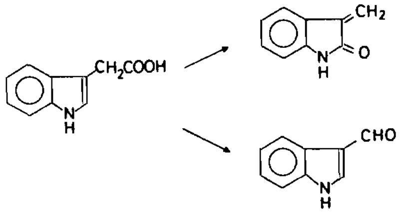
Received April 5, 1979

Contrary to previous claims it is found that aqueous $FeCl_3$ is at best a rather feeble catalyst for air oxidation of indole-3-acetic acid (IAA). Although Fe(III) does appear to undergo direct one-electron reduction by IAA under anaerobic conditions, the rate of the reaction is slow and comparable to the rate at which O_2 reacts with IAA. Ceric ion reacts very rapidly with IAA to produce 3-methylene-oxindole in the presence of air and an uncharacterised red, polymeric product in the absence of air. Production of the red polymer consumes two equivalents of either Fe(III) or Ce(IV) per IAA reacted. It is concluded that these systems are not good models for the peroxidase catalysed oxidation of IAA.

Introduction

The peroxidase catalysed oxidation of indole-3-acetic acid (IAA) by dioxygen has attracted considerable interest since it represents a fairly unusual case where peroxidases operate on dioxygen as a substrate rather than the more usual substrate, hydrogen peroxide. Much of the early work on this reaction was devoted to establishing the stoichiometry and identity of the products [1–5] but more recently attention has been turned to the application of physico-chemical methods to the investigation of the finer details of the reaction mechanism [6, 7]. It is now generally accepted that the primary reactions in the enzyme system lead to the products illustrated in (1). The production of 3-methyleneoxindole (MO) is favoured by low enzyme concentration and the indole-3-aldehyde by high enzyme concentration [3].



(1)

The idea that the initiation of IAA oxidation involved a simple electron transfer to the Fe(III) of the peroxidase heme was first seriously proposed by Hinman and Lang [1]. This assertion was in part supported by observations on oxidations of IAA and some of its derivatives induced by a number of inorganic oxidants, including aqueous Fe(III). The assertion of one-electron transfer as the key step was later corroborated by the report of Ricard and Job that ferroperoxidase carbonyl was formed when ferriperoxidase was treated with IAA under an atmosphere of CO [6]. The latter authors also confirmed the observation of Hinman and Lang that aqueous ferric chloride catalysed the oxidation of IAA by dioxygen to 3-methyleneoxindole, thereby entrenching this system as a good inorganic model for the peroxidase IAA system.

More recently the validity of the observation of ferroperoxidase carbonyl formation from ferriperoxidase, IAA and CO has been questioned both on theoretical and experimental grounds [8]. It therefore seemed appropriate to reexamine the validity of the aqueous $FeCl_3$ model, particularly with respect to separating the roles of Fe(III) and O_2 as oxidants and determining the possible role of trace impurities, such as peroxides, on the reaction. The present paper describes the results of such a reexamination.

Results and Discussion

A series of preliminary experiments were performed to establish (i) the importance of organic impurities on the reaction of aqueous $FeCl_3$ with IAA in the presence, or absence of O_2, (ii) whether aqueous $FeCl_3$ really catalyses the autoxidation of IAA and (iii) whether aqueous $FeCl_3$ is reduced by IAA in the absence of O_2.

Most of the experiments described below were performed in duplicate with substrates of reagent grade straight from the bottle and with substrates that were carefully recrystallised several times under inert atmosphere. In no case was any significant difference observed and it is concluded that the

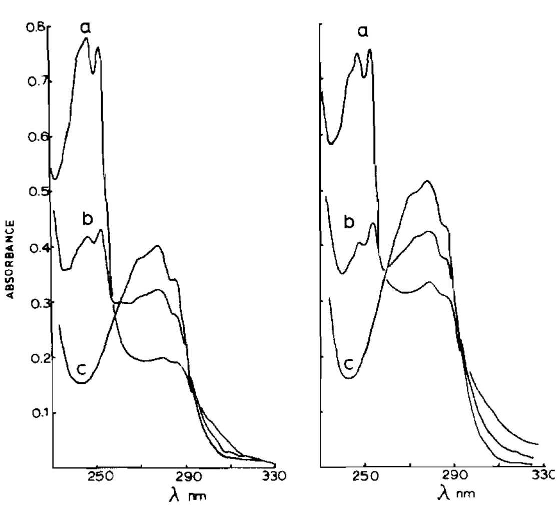


Fig. 1. Aerobic autoxidation of IAA in the presence (l.h. spectra) and absence (r.h. spectra) of Fe(III). Reaction time: a) 45 h, b) 5 h, and c) 0 h. T = 30 °C. [$FeCl_3$] = 1.1×10^{-5} *M*, [IAA] = 10^{-4} *M*. Acetate buffer at pH 5.

reactions to be described are relatively insensitive to organic impurities.

In order to establish the effectiveness of $FeCl_3$ as a catalyst for the autoxidation of IAA the experiments described by Hinman and Lang [1] were repeated. To our surprise the rates of autoxidation in the presence and absence of $FeCl_3$ were not very different and although the reaction was slightly faster in the presence of $FeCl_3$ the difference was hardly great enough to suggest effective catalysis (see Fig. 1). The same was true over a wide range of $FeCl_3$ and IAA concentrations and over the pH range 2.0–5.0. Similar results were obtained with 1-methylindole-3-acetic acid (MIAA), the rate being about fifty per cent faster in the presence of $FeCl_3$ (10^{-5} *M*) than in its absence.

In Fig. 2 the rates of reactions of IAA with oxygen in the absence and presence of some oxidising metal ions is compared. Rather surprisingly, since copper complexes are often very effective autoxidation catalysts, the Cu(II) species seem to suppress autoxidation of IAA to MO. On the other hand the strongly oxidising Ce(IV) species dramatically increase the rate of production of MO.

Reactions such as those shown in Fig. 2 were also carried out in the complete absence of oxygen. Very little change was observed in the uv/visible spectra and on the basis of that evidence alone it could have been concluded that no reaction was occurring. However, in the case of Ce(IV) the reaction mixture immediately became turbid and pink in colour, clearly showing the occurrence of a fast reaction. Similar changes occurred in the Fe(III) reaction but much more slowly.

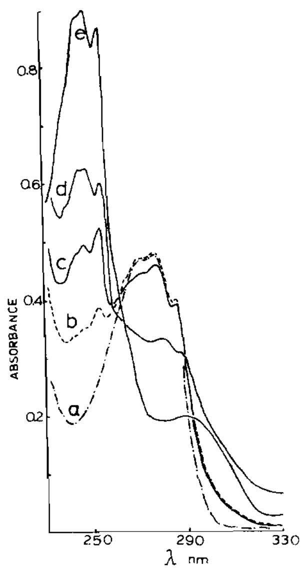


Fig. 2. Aerobic reactions of IAA with some oxidising metal ions. a) Reaction time zero, b) after 45 h with [$CuCl_2$] = 2×10^{-4} *M*, c) after 45 h with no added metal ion, d) after 45 h with [$FeCl_3$] = 2×10^{-4} *M*, e) after 5 min with [$Ce_2(SO_4)_3$] = 2×10^{-4} *M*. T = 25 °C. Bisulphate buffer at pH 2.

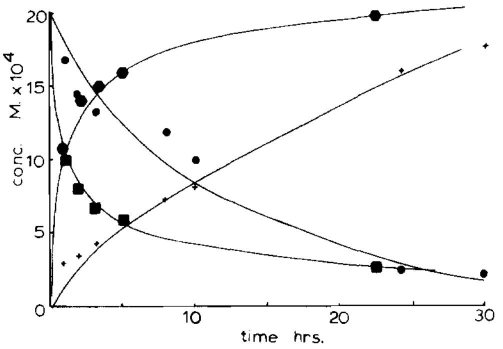


Fig. 3. Anaerobic reduction of Fe(III) by IAA and MIAA. T = 30 °C. [Indole] = 10^{-3} *M*. [$FeCl_3$]$_{initial}$ = 2×10^{-3} *M*. Bisulphate buffer at pH 2. •: [Fe(III)] and +: [Fe(II)] for IAA reaction. ■: [Fe(III)] and ⬢: [Fe(II)] for MIAA reaction.

Since the spectra of the organic species gave little information on what, if any, reaction was occurring, the fate of the metal ion was studied instead. The changing concentrations of Fe(III) and Fe(II) in anaerobic reactions with IAA and MIAA are shown in Fig. 3. In these experiments it is clear that the Fe(III) is being directly, albeit slowly, reduced to Fe(II) by the indolic reactants. It is also clear that the reactions consume close to two equivalents of Fe(III) per equivalent of indole reacted. Although ceric ion behaved in the same manner the reactions were too fast to follow their progress by chemical means. It was also observed that no peak appeared at 255 nm (MO) when the products of the anearobic reactions were exposed to air, provided the metal ion oxidant was in a ratio equal to, or in excess of, 2:1 with respect to indole. When the metal ion was present in less than 2:1 ratio the appearance of some MO was observed on exposure of anearobic reaction products to air.

From the foregoing observations, the following conclusions may be drawn: i) aqueous Fe(III) can directly oxidise IAA and MIAA without the intervention of oxygen, 2) the final product is two oxidation equivalents removed from the reactant and 3) the persistence of typical indole bands at *ca.* 280 nm indicates the presence of intact indole units in the reaction products. The bands at 280 nm are more intense in the products than the reactants, but they appear to remain almost unchanged during the anaerobic reaction due to the removal of Fe(III) or Ce(IV) which absorb quite strongly in the same region.

Further attempts to characterise the organic reaction product were no more successful than those reported previously [1]. The intense red colour of the product at $pH < 6$ indicates the presence of a chromophore arising from major disruption of the indole conjugation. Although the red material could be fractionated by selective solvent extraction, there was no identifiable chemical difference between the fractions. It seems likely therefore that the product is a copolymer containing some indolic units and others where the indole conjugation has been altered. An oxidative coupling polymerisation of the stoichiometry shown in (1) seems likely:

$$n\,C_{10}H_9O_2N \rightarrow [C_{10}H_7O_2N]_n + 2nH^+ + 2\,ne^- \quad (1)$$

Infrared spectra of the reaction products were consistently of poor quality despite the application of a number of sample preparation techniques. All bands were very broad, but the most prominent was at 1700 cm^{-1}, suggesting the continued presence of at least some carboxylate groups in the product.

The present observations all fit rather well with the interpretations of Hinman and Lang of the mechanism of non-enzymatic oxidation of IAA and there remains no doubt that aqueous Fe(III) can effect a one-electron extraction from IAA, or its close derivatives. In the presence of oxygen the cation radical produced by such a progress is rather efficiently intercepted by molecular oxygen with the ultimate production of 3-methyleneoxindole. The efficiency of this trapping is clearly manifest in the aerobic oxidation of IAA in the presence of Ce(IV). The reaction of Ce(IV) with IAA is much faster than the spontaneous autoxidation of IAA yet the main product in the presence of air is MO. In the case of Fe(III) the metal ion oxidation is comparable in rate to the spontaneous autoxidation. In this case the aerobic reaction products probably result partly from intervention of Fe(III) and partly from direct reaction of IAA with oxygen. Both processes lead predominantly to MO production at low concentration of metal ion and IAA. At higher concentrations ($\gtrsim$·001 *M*) the efficiency of interception by oxygen declines, due to the limited concentration of oxygen in water, and polymeric products predominate.

In the light of the above information we conclude that dilute aqueous Fe(III) is not a particularly useful model for peroxidase. Most peroxidases have standard potentials of <0 mV. We have previously argued that this is much too low to expect them to oxidise indoles by direct electron transfer, an assertion which was born out by experiment [8]. Even $Fe(CN)_6^{3-}$ ($E_o \sim +400$ mV at pH = 7) does not oxidise IAA. That aqueous Fe(III) in the absence of strongly complexing ligands ($E_o \sim +700$ mV) should oxidise IAA slowly is not surprising and that Ce(IV) should effect rapid oxidation ($E_o \sim +1{,}700$ mV) is entirely expected. However, a valid model for the peroxidase IAA system should satisfy the condition that IAA not be anaerobically oxidised by the complex, but be rapidly oxidised in the presence of both complex and oxygen.

Experimental

Materials

Indole-3-acetic acid was obtained as reagent grade material from Aldrich Chemical Co. High purity material was prepared by cycling through several recrystallisations from boiling water under nitrogen and finally drying under high vacuum at room temperature.

1-methylindole-3-acetic acid was prepared by a modification of the method of Snyder and Eliel [9]. A number of different literature syntheses were attempted, but a great deal of difficulty was experienced obtaining a product relatively free from impurity. The method finally used was as follows: Sodium metal (1.45 g) was added in small pieces to liquid ammonia (100 ml) contained in a 3-neck flask continually flushed with N_2 and cooled in an acetone/dry ice bath. The blue solution was stirred vigorously for 30 min and then a slurry of indole-3-acetic acid (5.0 g) in peroxide free ether (20 ml) was added. After further stirring (10 min) methyl iodide (15 g) was added dropwise over a period of 2 hours. The ammonia was then allowed to evaporate at room temperature and the residue was extracted with aqueous methanol (100 ml, 50 per cent v/v). The extract was filtered and the methanol evaporated. The aqueous residue was acidified with dilute HCl to pH = 4 and cooled in an ice bath. The crystals were collected, dried and redissolved in ethanol (75 ml). Water was added until cloudiness just persisted and the solution was left at –4° overnight. The white needles (3.0 g) were still visibly contaminated with pink flecks. Repeated recrystallisations from benzene/petroleum ether gave a product which showed no impurities by H^1 nmr (500 mg; m.p. 126–127 °C, lit. 127–129 °C [9] *).

Reactions of Indoles with $FeCl_3$ under Nitrogen

All solutions were prepared under N_2 using deoxygenated water, prepared by distillation under oxygen-free nitrogen, and were transferred from vessel to vessel by extraction through serum cap with hypodermic syringes. Samples for analysis were extracted by syringe. Ultraviolet spectra were measured by injection of samples into nitrogen filled cuvettes containing, when necessary for purposes of dilution, a buffer solution identical to that of the reaction mixture.

Ferric ion was analysed spectrophotometrically as its thiocyanate complex by comparing its absorption at 482 nm with a calibration curve prepared from a series of solutions of known concentration of Fe(III) and with the same buffer composition as the reaction mixture. A similar procedure was employed to determine the Fe(II) concentration as the 1,10-phenanthroline complex.

Acknowledgement

Financial support for this work from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

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*Even our crude material, which contained *ca.* 20 per cent impurities by n.m.r. had a m.p. of 128–29 °C.