RESEARCH COMMUNICATION

Electrospray mass-spectrometric, spectrophotometric and electrochemical methods do not provide evidence for the binding of nitric oxide by pyocyanine at pH 7

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In several recent publications on pyocyanine, its mechanism of action has been attributed to an ability to react with nitric oxide (NO), resulting in the formation of an adduct. We examined the chemical interaction of pyocyanine and NO using electrospray (ES) MS, spectrophotometry and voltammetry at neutral pH

and with 10–100 μ M pyocyanine. No binding of NO to pyocyanine was observed. Alternative mechanisms for the inhibition of NO-induced vasorelaxation by pyocyanine should be sought.

INTRODUCTION

Pyocyanine (Pyo) is a water-soluble blue pigment produced in large quantities by active cultures of the Gram-negative bacterium *Pseudomonas aeruginosa*. Pyocyanine has a variety of pharmacological effects on eukaryotic and prokaryotic cells. Pyo has been shown to inhibit the ciliary function of respiratory epithelial cells and to block bradykinin-induced endothelium-derived relaxation factor/nitric oxide (NO) release and vasorelaxation. Bozinovski and co-workers [1] reported that Pyo inhibits NO synthesis and blocks the relaxation of rabbit aortic smooth muscle induced by NO and organic nitrates. The mechanism by which Pyo interacts with NO both *in vitro* and *in vivo* is not known.

Warren and co-workers [2], in studies of the reaction between Pyo and NO, found that when NO was passed into a deoxygenated aqueous solution of Pyo, the initial blue colour was rapidly changed to pink. Fast atom bombardment (FAB) MS showed that the Pyo had completely disappeared. Desorption electron-impact ionization showed the presence of small amounts of 1-hydroxyphenazine and 1-methoxyphenazine, together with major ions at m/z 221 and 193. It was suggested these latter ions could be due to nitrosylation of Pyo by NO, followed by formation of a substituted oxazoline by dehydration, which could have occurred during the MS analysis. These results led to the speculation that a reaction between Pyo and NO may also occur *in vivo*, for example, in patients infected with *P. aeruginosa*. Removal of NO by reaction with Pyo could explain the inhibitory effect of Pyo on NO-induced vasodilation, and its interaction with NO therefore deserves further study.

A mechanism involving removal of NO by reaction with Pyo was not supported by the results of an exploratory experiment carried out in our laboratory in which NO in a closed aqueous system was analysed by a headspace-gas method [3]. Thus 124 pmol of NO injected into a Krebs solution, pH 7.4, showed NO recovery of $101 \pm 8.4\%$ (S.D.) in the absence of Pyo and $102 \pm 6.6\%$ in the presence of 10 μ M Pyo. This observation of the persistence of NO is inconsistent with the mechanism proposed by Warren and co-workers [2] involving the irreversible reaction between Pyo and NO. Our further investigations reported here reveal that, at physiological pH, Pyo does not react with NO; at lower pH, however, this reaction does occur.

MATERIALS AND METHODS

Materials

Pyocyanine was obtained from two sources; it was synthesized in-house by the method of Knight et al. [4] and was obtained commercially from Colour Your Enzyme (Bath, Ontario, Canada). The results obtained with material from the two sources were identical. All solutions were prepared in deionized water (Barnstead Nanopure®; Fisher Scientific Ltd., Ottawa, Ontario, Canada). NO (5.00% NO in N₂) was obtained from Scott Specialty Gases Inc. (Troy, MI, U.S.A.). A stock 1.0 M ammonium acetate buffer, pH 7.0, was prepared from reagent-grade ammonia and 100% acetic acid (BDH Chemicals Canada Ltd., Toronto, Ontario, Canada). A pH 7.0, NH₃/NH₄Cl (1.0 M) buffer was prepared from HCl and ammonia.

Methods

The pH measurements were made with an Accumet® Model 15 pH-meter (Fisher Scientific) calibrated with certified buffer solutions (Fisher Scientific, Nepean, Ontario, Canada) of pH 4.00, 7.00 and 10.00. Spectrophotometric studies were carried out with a Hewlett-Packard 8452A diode-array spectrophotometer using 1.0 cm quartz cells over the wavelength range 190–820 nm and an integration time of 1.0 s. The mass spectrometer (VG Quattro; Fisons Instruments, Altrincham, U.K.) was equipped with interchangeable ion sources for operation in ES and fast-ion-bombardment (FIB) modes.

Solutions were introduced into the ES source using a Harvard syringe pump at a flow rate of 17 μ l/min. FIB analyses were carried out with Pyo dispersed in a nitrobenzyl alcohol matrix. The identities of the ions produced by both ionization methods were investigated by collision-assisted dissociation (CAD) using argon as collision gas in the central sector of the triple-sector instrument. The polarographic analyser (model PAR 174 A; EG&G Instruments, Princeton Applied Research, Princeton,

Abbreviations used: Pyo, pyocyanine; NO, nitric oxide; FAB, fast atom bombardment; FIB, fast ion bombardment; ES, electrospray; CAD, collision-assisted dissociation; SQ, semiquinoid; a.m.u., atomic mass units.

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NJ, U.S.A.) employed a static mercury drop electrode unit (model 303), which contained a platinum auxiliary electrode, an Ag/AgCl/saturated aqueous KCl reference electrode, a hanging mercury drop electrode as the working electrode and a 15 ml quartz electrolytic cell. Further details regarding electrochemical instrumentation are given elsewhere [5,9]. Spectrophotometric and MS experiments were carried out with solutions exposed to the air and also with solutions deoxygenated by purging with N_2 . The electrochemical experiments were carried out only with oxygen-free solutions.

RESULTS

A 100 μ M unbuffered aqueous solution of Pyo had a pH of 6.3 and was blue in colour (Figure 1a). The UV-visible spectrum has

Figure 1 The UV-visible spectra of 100 μ M Pyo

(a) Unbuffered solution; (b) unbuffered solution after purging with 5.00% NO for 5 min; (c) as for (b), except that the solution was left for 1 h; (d) buffered with 200 mM ammonium acetate after purging with 5.00% NO for 5 min.

absorption maxima at 690, 379, 312 and 239 nm. The Pyo solution was purged, first with N_2 to deoxygenate it (resulting in no spectral change) and then for 5 min with the NO/ N_2 mixture. The colour of the solution became red immediately upon introduction of the NO and the pH fell to 4.0 after 5 min. The UV-visible spectrum had changed (Figure 1b), with absorption maxima at 525, 379 and 278 nm. Over the next hour, the pH remained stable at 4.0, but the colour of the solution gradually changed from red to a 'muddy' orange as two new absorption bands appeared at 433 and 370 nm (Figure 1c). These colour changes and the absorption bands for Pyo have been described in detail by Friedheim and Michaelis [6] and by Zaugg [7] respectively. The blue colour is due to Pyo, and the red colour, observed only in acidic solution, is due to protonated Pyo (PyoH⁺). The 'muddy' orange colour is due to a mixture of PyoH⁺ and the green semiquinoid (SQ) form of Pyo. The structures of the four species responsible for the colours are shown in Figure 2.

When the blue 100 μ M Pyo solution was buffered at pH 7.0 with 200 mM ammonium acetate, purging with NO/ N_2 did not induce a change in pH, and no colour change was observed (Figure 1d). The only change noted was the appearance of two bands of extremely low intensity at 433 and 370 nm. These bands, as noted above, are characteristic of the SQ form of Pyo.

ES ionization MS of unbuffered aq. 10 μ M Pyo solution (pH 6.3) gave the spectrum shown in Figure 3a. PyoH⁺ (m/z 211) is the major peak, with smaller unidentified peaks at m/z 227 and at m/z 197. The former might be due to a hydroxylated impurity, and the latter might be due either to the presence of a small amount of 1-hydroxyphenazine or to collisional decomposition of PyoH⁺ in the ES source. The same spectrum was obtained for a 200 μ M Pyo solution, and the CAD spectra of both m/z 211 and 227 showed loss of 15 (CH_3), 28 (CO) and 43 (CH_3CO ?) atomic mass units (a.m.u.). When NO was bubbled into the unbuffered 10 μ M Pyo solution, m/z 227 and 197 decreased in intensity relative to m/z 211 and several small peaks appeared (Figure 3b). Of particular note in this Figure is the small peak at m/z 240. The relative intensity of this peak increased with time until after 35 min it was almost equal in height to m/z 211 (Figure 3c), and after 1 h was the dominant ion in the spectrum. This ion was identified by CAD as the NO⁺ adduct of pyocyanine. At low collision energy, 10 eV centre of mass, there was loss of 30 a.m.u. (NO) to give m/z 210 (Pyo⁺) and with increased energy, further fragmentation by loss of 15, 28 and 43 a.m.u. (Figure 4).

When a 10 μ M Pyo solution was buffered at pH 7.0 with 200 mM ammonium acetate (Krebs buffer cannot be used in a mass spectrometer) and bubbled with NO for 5 min, the solution was blue and PyoH⁺ was the dominant peak and the other major

Figure 2 The Pyo species

I, Pyo, blue; II, protonated Pyo (PyoH⁺), red; III, SQ form of Pyo, green; IV, Pyo-NO adduct.

Figure 3 ES ionization mass spectra of 10 μ M Pyo

(a) Unbuffered solution; (b) unbuffered solution after purging with 5.00% NO for 5 min; (c) as for (b), except that the solution was left for 35 min; (d) buffered with 200 mM ammonium acetate after purging with 5.00% NO for 5 min.

peak, 10 % of the intensity of PyoH^+ , was at m/z 287 (Figure 3d). There was no change in the spectrum in 22 min, and no formation of m/z 240. When the 10 μ M pyocyanine solution was first purged with NO/ N_2 and then immediately buffered at pH 7.0, no m/z 240 was observed nor was any found to build up. When the purged solution was allowed to stand for 1 h before buffering, then m/z 240 was present.

In an attempt to replicate the FAB results of Warren et al. [2], we carried out an FIB experiment using glycerol as the matrix. The major ion in the FIB spectrum was PyoH^+ . The spectrum was unusual in that m/z 212 was much too large to be the ^{13}C

isotope peak. This anomalous intensity, which has been previously noted in the thermospray mass spectrum of Pyo [8], appears to be a function of Pyo concentration and is not relevant to this discussion. Bathing the FIB probe in NO for 3 min had no effect on the spectrum, and no adduct was observed. The diffusion of NO into the FIB matrix is probably too slow for any significant chemistry to occur.

Our previous electrochemical studies on the Pyo redox properties [9] have been extended to the Pyo/NO system. A direct voltammetric experiment was carried out in 10 ml of a thoroughly deoxygenated solution of 10 μ M Pyo in 10 mM $\text{NH}_3/\text{NH}_4\text{Cl}$

Figure 4 The CAD spectrum of the Pyo-NO⁺ adduct

The collision energy was 10 eV (centre of mass).

buffer, pH 7.0. Application of the cathodic (negative-going) potential scan from 0.00 V to -0.50 V on a freshly dispensed mercury drop gave a well-defined Pyo reduction peak at -0.20 V. Purging the solution with 5.00 % NO (in 95 % N₂) changed the Pyo solution from faint blue to faint pink, which coincided with a pH change from 7.0 to 4.3 and an expected 0.20 V anodic shift of the Pyo reduction peak [10], which became broad and asymmetrical. Further addition of NH₃/NH₄Cl buffer (total 20 mM) changed the solution from faint pink to faint blue, raised the pH from 4.3 to 7.0 and restored the symmetrical Pyo reduction peak at -0.20 V, which was about 25 % smaller than the initial peak. An observed decrease in the peak current (height) is attributed mainly to the increased buffer concentration [9] and, to a lesser extent, to the possible loss of Pyo in an unidentified Pyo/NO reaction in acidic medium. Similar experiments carried out at pH 7.0 in well-buffered aqueous Pyo solutions, purged with NO/N₂, did not show any change in colour, Pyo reduction peak potential shift or Pyo reduction peak size.

DISCUSSION

The data obtained with three different experimental techniques provide a consistent picture of the effect of NO on aqueous solutions of Pyo. This bacterial pigment behaves as an acid–base indicator, being blue in its basic form (Pyo) and pink in its acidic form (PyoH⁺). The pK_a value is ≈ 5.5 [6]. The immediate colour change upon the introduction of NO to a supposedly deoxygenated, unbuffered Pyo solution is attributable to the immediate decrease in pH as PyoH⁺ becomes the dominant form. The presence of PyoH⁺ is confirmed by its UV–visible spectrum and the voltammetric results. Warren and co-workers [2] observed

the immediate colour change, but found no change in pH. We can offer no explanation for this difference. The colour changes of Pyo with change of pH have been observed previously [6,7]. Although the present experiments have not addressed the mechanism whereby NO bubbled into water results in the formation of H⁺, other investigators have provided a plausible explanation [11–13]. The formation of H⁺ when NO is bubbled into water is ascribed to the following reaction:

The occurrence of this reaction ($k = 2.1 \times 10^{-6} \text{ M}^{-2} \cdot \text{s}^{-1}$ [12]) implies either that none of our solutions was completely deoxygenated (the N₂ used for deoxygenation contained < 3 p.p.m. O₂) or that the NO/N₂ mixture itself contained traces of oxygen.

The ES mass spectra show that an NO/Pyo (m/z 240) adduct is formed at pH 4, but only very slowly. However, at long reaction times, the adduct appears to be the major component of the solution. The CAD spectrum of this ion is consistent with nitrosylation of Pyo in solution, as suggested by Warren and co-workers [2], but we did not observe dehydration in the sampling process as they report. The FIB spectra, although providing no important data, did show the problem of working with high concentration of Pyo in MS studies. The evidence is strong that Pyo-NO⁺ actually exists as such in solution, and is not formed by the ionization of Pyo-NO in the spraying process. This would imply the possible existence of the transient NO⁺ [14] in these solutions.

The biologically relevant observation made in the present work is that all three analytical methods are consistent in finding that Pyo is very stable at pH 7.0, showing essentially no evidence of reaction or interaction with NO. The only hint of a possible interaction was the observation of the two weak absorption

bands (Figure 1d) that appear to be due to the formation of a very small amount of the SQ. Since the intensities of the bands did not change with time, their presence does not denote a reaction of any significance. It should be noted that the experimental conditions used by Warren et al. [2] differed from some of those used in the present study; in particular, our Pyo concentrations were much lower, in the micromolar range, and we experimented with both buffered and unbuffered systems.

Our results show that NO can slowly form a complex with Pyo in acidic solution, but certainly not at pH 7.0. This leads to the conclusion that the inhibitory effect of Pyo on NO-induced vasorelaxation cannot be explained by interaction between Pyo and NO, resulting in an adduct formation. This may be important to investigators working on NO biological problems who have heretofore attributed an effect of Pyo to its chemical interaction with NO. Other mechanisms by which Pyo inhibits NO-induced vasorelaxation could include direct inhibition of NO synthase, which has been shown to occur for guinea-pig hippocampal constitutive NO synthase [15]. This and other potential mechanisms have yet to be fully explored.

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