The *Pseudomonas aeruginosa* Secretory Product Pyocyanin Inactivates α₁ Protease Inhibitor: Implications for the Pathogenesis of Cystic Fibrosis Lung Disease

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α₁ Protease inhibitor (α₁PI) modulates serine protease activity in the lung. Reactive oxygen species inactivate α₁PI, and this process has been implicated in the pathogenesis of a variety of forms of lung injury. An imbalance of protease-antiprotease activity is also detected in the airways of patients with cystic fibrosis-associated lung disease who are infected with *Pseudomonas aeruginosa*. *P. aeruginosa* secretes pyocyanin, which, through its ability to redox cycle, induces cells to generate reactive oxygen species. We tested the hypothesis that redox cycling of pyocyanin could lead to inactivation of α₁PI. When α₁PI was exposed to NADH and pyocyanin, a combination that results in superoxide production, α₁PI lost its ability to form an inhibitory complex with both porcine pancreatic elastase (PPE) and trypsin. Similarly, addition of pyocyanin to cultures of human airway epithelial cells to which α₁PI was also added resulted in a loss of the ability of α₁PI to form a complex with PPE or trypsin. Neither superoxide dismutase, catalase, nor dimethylthiourea nor depletion of the media of O₂⁻ to prevent formation of reactive oxygen species blocked pyocyanin-mediated inactivation of α₁PI. These data raise the possibility that a direct interaction between reduced pyocyanin and α₁PI is involved in the process. Consistent with this possibility, pretreatment of α₁PI with the reducing agent β-mercaptopoethanol also inhibited binding of trypsin to α₁PI. These data suggest that pyocyanin could contribute to lung injury in the *P. aeruginosa*-infected airway of cystic fibrosis patients by decreasing the ability of α₁PI to control the local activity of serine proteases.

Tight regulation of local protease activity is critical to the maintenance of the physiologic function of the lung and other tissue sites (15, 40, 48). Serine proteases such as neutrophil elastase are among the proteases found within the human airway (15, 16, 20, 38, 40). Serine protease activity must be tightly regulated in order to protect local tissue from protease-mediated injury. This is accomplished in vivo through the presence of a group of proteins which specifically inhibit serine protease activity (32, 40, 48). Among the key antiproteases in the airway is α₁ protease inhibitor (α₁PI) (32, 40, 48). α₁PI is a 51-kDa protein member of the serpin class of serine protease inhibitors (32, 40, 48). α₁PI forms a nearly irreversible enzymatically inactive complex with various serine proteases, including human neutrophil elastase (HNE), porcine pancreatic elastase (PPE), and trypsin (29, 40, 48). The active site of α₁PI provides a putative cleavage site for the target enzymes, and it is the methionine 358 within that location which is key for both the protease specificity and inhibitory activity of α₁PI. Modifications of this methionine markedly decrease the inhibitory activity of α₁PI (29, 40, 48).

A number of laboratories have shown that exposure of α₁PI to various oxidant sources, including activated phagocytes (6, 14, 28, 34, 39, 50) and cigarette smoke (17, 41), results in rapid inactivation of the protein due to oxidation of methionine 358 (31). Since the hereditary decrease in α₁PI activity leads to early-onset emphysema (10), it has been postulated that oxidant-mediated inactivation of α₁PI, resulting in decreased local regulation of serine protease activity, is an important contributor to the pathophysiology of cigarette-associated chronic obstructive lung disease (6, 30).

An imbalance of protease-antiprotease activity is also detected in the airways of patients with cystic fibrosis-associated lung disease (5, 16, 18, 19, 35). Most of this elevated protease activity is due to an increase in HNE activity (23, 43, 45). Studies indicate that the unbalanced protease imbalances are a result of both elevated amounts of HNE and the presence of functionally inactive α₁PI (1, 2, 5, 35, 38, 44). The reason for the presence of inactive α₁PI is unclear.

The onset of progressive lung disease in cystic fibrosis usually coincides with the development of persistent colonization and infection of the airway with *Pseudomonas aeruginosa*. *P. aeruginosa* secretes a number of potentially cytotoxic factors, including the phenazine derivative pyocyanin (21, 24, 25, 51). Under aerobic conditions, exposure of pyocyanin to NADH or other cell-derived reducing equivalents, leads to redox cycling of the compound with resultant formation of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) (4, 11, 22, 24, 25). Given the ability of pyocyanin to induce oxidant production, we hypothesized that this *P. aeruginosa*-derived product could contribute to the pathogenesis of the protease-mediated component of cystic fibrosis lung disease by serving as an additional source of oxidant-mediated inactivation of α₁PI. The in vitro work reported herein supports the ability of redox cycling of pyocyanin to inactivate α₁PI. However, the mechanism may not directly involve oxidant production.

**MATERIALS AND METHODS**

Reagents. NADH, CuZn superoxide dismutase (SOD), trypsin, porcine pancreatic elastase (PPE), H₂O₂, methionine, histidine, catalase, and dimethylthiourea (DMTU) were obtained from Sigma Chemical Co., St. Louis, Mo. α₁PI was...
RESULTS

Pyocyanin-NADH blocks \( \alpha_{1}PI \)-protease complex formation. Several laboratories, including our own, have shown that addition of NADH to pyocyanin leads to the reduction of pyocyanin to the pyocyanin radical (4, 11, 22, 24–26). Under aerobic conditions, the pyocyanin radical will transfer an electron to \( O_2 \), leading to the formation of \( O_2^- \) (4, 11, 22, 24–26). Consistent with these earlier data (4, 11, 22, 24–26), when NADH and pyocyanin were incubated under conditions in which \( O_2 \) was previously depleted from the system by \( N_2 \) bubbling, an EPR spectrum indicative of the pyocyanin radical was detected (data not shown). When the same reaction was performed under aerobic conditions, the pyocyanin radical was no longer seen, but \( O_2^- \) production was readily detected by DMPO spin trapping (data not shown).

Given the susceptibility of \( \alpha_{1}PI \) to inactivation by oxidant species (6, 17, 41, 52), the ability of NADH-mediated redox cycling of pyocyanin to decrease \( \alpha_{1}PI \) activity was assessed. As shown in Fig. 1 and 2, the presence of NADH and pyocyanin decreased the ability of \( \alpha_{1}PI \) to form a stable complex with either trypsin or PPE. Concentrations of pyocyanin required to completely prevent detectable complex formation varied from experiment to experiment, ranging from 25 to 125 \( \mu \)M. In the PPE experiments, a major protease-\( \alpha_{1}PI \) complex (top) and a somewhat fainter protease-\( \alpha_{1}PI \) complex (bottom) were routinely observed. The lower bands are likely due to the presence of chymotrypsin and/or trypsin, which routinely contaminate commercial PPE preparations and also form a complex with \( \alpha_{1}PI \). Neither NADH nor pyocyanin alone prevented formation of the \( \alpha_{1}PI \)-protease complex (data not shown), indicating that reduction of pyocyanin was required. These results were similar, regardless of whether the \( \alpha_{1}PI \)-protease complex was detected by silver staining or immunoblot analysis.

An additional feature frequently noted when the NADH-

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Fig. 2. Immunoblot with antisera to \( \alpha_{1}PI \). The gel sample in lane 1 consisted of \( \alpha_{1}PI \) which had been incubated for 30 min at 37°C with 200 \( \mu \)g of PPE. Lane 2 shows results obtained under the same conditions as lane 1, except that 6 \( mM \) NADH and 100 \( \mu \)M pyocyanin were added prior to the addition of PPE to the reaction mixture. The results are representative of five separate experiments.
pyocyanin combination was present was a decrease in the apparent quantity of α1PI detectable on the gel and evidence of proteolytic degradation products of α1PI (Fig. 1 and 2). The fact that these were detected by anti-α1PI immunoblotting indicates they indeed reflect degradation of α1PI. Lower-molecular-weight bands reactive with anti-α1PI were not observed with NADH-pyocyanin-treated α1PI unless an active protease was also added to the reaction mixture (data not shown [n = 3]). These data are consistent with the hypothesis that NADH-pyocyanin exposure leads to a modification of α1PI, such that the antiprotease is converted to a legitimate substrate for PPE or trypsin.

Since the incubation conditions described above resulted in exposure of trypsin and PPE to NADH-pyocyanin as well as α1PI, experiments were performed so that we could be certain that the effect of NADH-pyocyanin was on α1PI and not the protease. α1PI-protease complex formation was compared under conditions in which α1PI or the protease was first exposed to NADH-pyocyanin for 30 min, after which, the protease or α1PI was then added to the system, respectively, and α1PI-protease complex formation was assessed (Fig. 3). When α1PI was first incubated with NADH-pyocyanin and then PPE or trypsin was added, no α1PI-protease complex was generated (Fig. 3). In contrast, when trypsin or PPE was the component incubated initially with NADH-pyocyanin, after which α1PI was added, the same magnitude of α1PI-protease complex was observed as in the non-NADH-pyocyanin-treated control (Fig. 3). These results indicate that the effect of NADH-pyocyanin is on α1PI rather than the protease.

**Biochemical evidence of NADH-pyocyanin inactivation of α1PI.** As confirmation that exposure to NADH-pyocyanin leads to loss of α1PI functional activity, the ability of NADH-pyocyanin-treated α1PI to inhibit PPE enzymatic activity was assessed. α1PI exposed to NADH-pyocyanin exhibited an impaired ability to inhibit PPE cleavage of succinyl-l-alanyl-l-alanyl-p-nitroanilide. In the absence of α1PI, cleavage of succinyl-l-alanyl-l-alanyl-p-nitroanilide by PPE yielded a decrease in A_{410} of 0.132 ± 0.012 U/min (mean ± standard error [SE]; n = 10). Addition of α1PI decreased this to 0.040 ± 0.005 U/min (mean ± SE; n = 10). When α1PI was first exposed to NADH-pyocyanin for 10 min, its ability to inhibit PPE activity was significantly decreased (P < 0.001 by analysis of variance) because A_{410} was 0.076 ± 0.009 U/min (mean ± SE; n = 10). NADH-pyocyanin had no effect on PPE activity as assessed in this assay (data not shown).

**Epithelial cell-mediated redox cycling of pyocyanin inactivates α1PI.** In vivo redox cycling of pyocyanin in the P. aerugi- nosa-infected airway would likely occur via epithelial cell-mediated reduction of the compound rather via its interaction with extracellular NADH or NADPH. We have previously shown that incubation of monolayers of A549 (3, 12, 37) or HBE cells (12) with pyocyanin results in the formation of O₂⁻, as detected by spin trapping. Since initial reduction of pyocy-

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**FIG. 3.** Immunoblot with antisera to α1PI in which gel samples were comprised of 10 μg of α1PI plus 200 μg of trypsin which had been incubated for 30 min at 37°C. Lanes 1 to 3 show the results obtained when α1PI was previously exposed to 6 mM NADH and 100 μM pyocyanin alone (lane 1), or with SOD (300 U/ml; lane 2) or catalase (5,000 U/ml; lane 3) added, for 20 min prior to the addition of trypsin to the reaction mixture. Samples in lanes 4 to 6 were obtained under the same conditions as those of lanes 1 to 3, except that the trypsin rather than α1PI was previously exposed to NADH and 100 μM pyocyanin alone (lane 4), or with SOD (300 U/ml; lane 5) or catalase (5,000 U/ml; lane 6) added, for 30 min prior to its interaction with α1PI. The results are representative of three experiments.
anin-induced \( \text{O}_2^- \) formation were responsible for NADH-pyocyanin-mediated inhibition of \( \alpha_\text{PI} \) activity. In order to test this, we determined if the presence of SOD, catalase, or the \( \text{H}_2\text{O}_2 \)-hydroxyl radical scavenger DMTU blocked NADH-pyocyanin-mediated inactivation of \( \alpha_\text{PI} \). Surprisingly, none of these agents alone nor the combination of SOD and catalase exhibited any ability to block the effect of NADH-pyocyanin (Fig. 3). SOD and catalase are reasonably large proteins, raising the possibility that they were unable to adequately reach the site of \( \alpha_\text{PI} \) which required protection. However, we found that methionine, which blocks MPO-mediated inactivation of \( \alpha_\text{PI} \) (6) via its ability to spare methionine 358 within the active site of \( \alpha_\text{PI} \) (6, 29, 40, 48), did not protect \( \alpha_\text{PI} \) from the effects of NADH-pyocyanin (data not shown). In addition, since \( \text{O}_2^- \) is not generated as a consequence of pyocyanin reduction under anaerobic conditions, we assessed the ability of \( \text{O}_2^- \) depletion on the process by bubbling the experimental system with \( \text{N}_2 \) prior to the addition of pyocyanin. NADH-pyocyanin retained its ability to inactivate \( \alpha_\text{PI} \) under \( \text{O}_2^- \)-depleted conditions (Fig. 6). That bubbling of \( \text{N}_2 \) effectively depleted the system of \( \text{O}_2^- \) was confirmed with the anaerobic indicator resazurin (33).

These data do not suggest a role for \( \text{O}_2^- \), or subsequently generated oxidants such as \( \text{H}_2\text{O}_2 \), in the ability of reduced pyocyanin to inactivate \( \alpha_\text{PI} \). Instead, they raise the possibility that pyocyanin radical, a reducing species formed by the initial electron transfer from NADH to pyocyanin, could be inactivating \( \alpha_\text{PI} \) via a reducing rather than an oxidizing reaction. In support of this, exposure of \( \alpha_\text{PI} \) to the reducing agent \( \text{β-mercaptoethanol} \) resulted in an inability of the \( \alpha_\text{PI} \) to subsequently bind trypsin (Fig. 7). This is in contrast to reports in the literature (7, 31, 46) and our own experience (data not shown) that, once formed, the \( \alpha_\text{PI} \)-trypsin complex does not dissociate upon exposure to \( \text{β-mercaptoethanol} \).

**DISCUSSION**

An elevated ratio of protease to antiprotease activity has been detected in the \( P. \text{aeruginosa} \)-infected airways of cystic fibrosis patients, and this is felt to contribute to the pathogenesis of cystic fibrosis lung disease (23, 43, 45). This imbalance is likely due to the presence of markedly elevated levels of the serine protease HNE as well as a higher than expected frequency of inactive \( \alpha_\text{PI} \) (1, 2, 5, 35, 38, 44). Given the known susceptibility of \( \alpha_\text{PI} \) to oxidant-mediated inactivation (6, 14, 17, 34, 41, 52), the ability of the redox-active \( P. \text{aeruginosa} \) secretory product pyocyanin to inactivate \( \alpha_\text{PI} \) was assessed. Such a process could contribute to protease-mediated lung injury in cystic fibrosis, as well as potentially necrotizing acute nosocomial \( P. \text{aeruginosa} \). Nearly all strains of \( P. \text{aeruginosa} \) are capable of producing pyocyanin.

In support of this hypothesis, we found that the reaction of NADH with concentrations of pyocyanin reported to be present in sputum sol of \( P. \text{aeruginosa} \)-infected cystic fibrosis patients (51), in excess of 100 \( \mu \text{M} \), was able to alter \( \alpha_\text{PI} \) such that it was no longer able to bind to and inhibit the enzymatic activity of the serine proteases PPE and trypsin (binding studies only). The concentration of pyocyanin present in the lung in the setting of \( P. \text{aeruginosa} \) pneumonia in the non-cystic fibrosis-hospitalized patient is not known. Our studies were performed with concentrations of \( \alpha_\text{PI} \) and serine proteases very close to the mean values of 12 and 88 \( \mu \text{M} \) of \( \alpha_\text{PI} \) and human neutrophil elastase, respectively, per ml reported to be present in the sputum of \( P. \text{aeruginosa} \)-infected cystic fibrosis patients (38). Although we would expect a similar effect of pyocyanin exposure on \( \alpha_\text{PI} \) binding to HNE, this was not assessed due to the cost-prohibitive requirement of HNE for such studies. \( \alpha_\text{PI} \) binding to all serine proteases occurs via the same basic process (32, 40, 48).

Similar to the results with NADH-pyocyanin, in studies in which human lung epithelial cell monolayers were exposed to pyocyanin, \( \alpha_\text{PI} \) was inactivated when the protein was present in the extracellular milieu. Thus, in spite of the intracellular site at which pyocyanin reduction likely occurs, there was sufficient interaction between reduced pyocyanin and/or its oxidant products to inactivate \( \alpha_\text{PI} \). Previous spin trapping studies with endothelial cells support the concept that these pyocyanin-derived products do have access to the extracellular space (4).

Regardless of whether NADH or epithelial cells were employed as the means of reducing pyocyanin, incubation of pyocyanin-treated \( \alpha_\text{PI} \) with either trypsin or PPE resulted in the formation of proteolytic degradation products of \( \alpha_\text{PI} \). This suggests that redox cycling of pyocyanin leads to modifications of \( \alpha_\text{PI} \) which allow it to be cleaved by the protease rather than

**FIG. 6.** Immunoblot with antisera to \( \alpha_\text{PI} \) in which gel samples were comprised of 10 \( \mu \text{g} \) of \( \alpha_\text{PI} \) alone (lane 1) or 10 \( \mu \text{g} \) of \( \alpha_\text{PI} \) alone plus 200 \( \mu \text{g} \) of trypsin (lane 2), which had been incubated for 30 min at 37°C. Lanes 3 and 4 show results obtained under the same conditions as lane 2, except that 6 \( \mu \text{M} \) NADH and 100 \( \mu \text{M} \) pyocyanin were added prior to the addition of trypsin to the reaction mixture. The incubation in lane 3 was performed under standard aerobic conditions, whereas lane 4 reflects results obtained under \( \text{O}_2 \)-depleted conditions (Fig. 6). That bubbling of \( \text{N}_2 \) effectively depleted the system of \( \text{O}_2^- \) was confirmed with the anaerobic indicator resazurin (33).
resulting in the formation of an irreversible complex between α1PI and the protease. Oxidant-mediated inactivation of another airway protease inhibitor, secretory leukocyte protease inhibitor (SLPI), also renders it more susceptible to proteolysis (49).

Consistent with our previous work (3, 4, 37) and that of others (4, 8, 11, 22, 24–26, 42), we found by using EPR techniques that the addition of NADH to pyocyanin leads to the initial formation of pyocyanin radical, which in the presence of O2 transfers electron to O2 to form O2·−. O2·− production was also observed upon the addition of pyocyanin to human airway epithelial cells. Although not specifically measured, production of O2·− would lead in turn to the formation of H2O2 via the dismutation reaction of O2·− with itself (36). In view of previous observations that oxidation of methionine 358 at the α1PI active site is the mechanism whereby MPO-H2O2 inactivates α1PI (29, 40, 48), we suspected a similar process was involved in the inactivation of α1PI by pyocyanin. However, several pieces of data argue against such a mechanism. Free methionine failed to protect α1PI from the effect of pyocyanin, in contrast to its reported ability to protect the protein from inactivation by the MPO-H2O2 system (6). Neither SOD, catalase, or DMTU, nor depletion of O2 from the system altered the ability of NADH-pyocyanin to inactivate α1PI. These data argue strongly against a role for pyocyanin-derived O2·− and/or H2O2 in the ability of this P. aeruginosa-derived compound to inactivate α1PI. Thus, pyocyanin-mediated inactivation of α1PI inhibition does not likely occur via oxidation of methionine at the α1PI active site.

These data raise the possibility that a direct interaction between α1PI and the pyocyanin radical itself, rather than reactive oxidant species generated from the interaction of reduced pyocyanin and O2·−, modifies α1PI such that it loses its ability to bind serine proteases. Under this hypothesis, the pyocyanin radical would directly modify α1PI by the transfer of an electron (reduction) to an amino acid constituent of the protein resulting in either direct modification of the active site or alternativelly a conformational change in the molecule that decreases its ability to irreversibly bind serine proteases. This is consistent with the fact that the pyocyanin radical is predominantly a reducing radical. Supporting this hypothesis, exposure to the reducing agent β-mercaptoethanol also resulted in a loss of the ability of α1PI to bind trypsin. We are unaware of studies investigating the potential for reduction rather than oxidation of α1PI to decrease its ability to bind serine proteases. What amino acid(s) would be the target of such reduction is difficult to predict. This hypothesis will need to be further examined with previously generated site-specific mutations of α1PI as well as by molecular analysis of the pyocyanin-modified protein.

Although the exact mechanism remains to be definitively defined, our data are consistent with the potential for pyocyanin present in the P. aeruginosa-infected airway to cause a local inhibition of α1PI activity, thereby contributing to protease-mediated tissue injury. It is likely that the pathophysiolog responsible for the protease-antiprotease imbalance which marks the airway of cystic fibrosis patients is multifactorial. There is an increased burden of HNE due to the large neutrophil infiltrate that marks this disease state. Oxidants produced by the same neutrophils, particularly those derived from MPO-H2O2, also likely serve to inactivate α1PI. Interestingly, recent data suggest that pyocyanin, through its ability to induce interleukin 8 release from airway epithelial cells, may contribute to this neutrophil infiltration of the airway (13). Besides α1PI, the airway contains other antiproteases, such as (SLPI), capable of inhibiting HNE (49). SLPI is also susceptible to oxidant-mediated inactivation (27, 49) due to the presence of a methionine residue at its active site. Whether SLPI can also be inactivated by pyocyanin is unknown and is worthy of further investigation.

In summary, the present work demonstrates that, once reduced, pyocyanin can in turn interact with α1PI in such a way that there is a loss of the protein’s ability to form an irreversible complex with and inhibit the enzymatic activity of serine proteases. Although the exact mechanism requires further delineation, occurrence of these events in vivo could contribute to the pathogenesis of serine protease-mediated injury in P. aeruginosa-infected lungs of patients with cystic fibrosis chronic lung disease and/or acute nosocomial P. aeruginosa pneumonia.

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REFERENCES

14. Döring, G., W. Goldstein, A. Röll, P. O. Schiötz, N. Høiby, and K. Botzen-